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13. ABSTRACT ***(Maximum 200 Words)***

The MMAC1/PTEN/TEP1 phosphatase tumor suppressor gene contributes to the initiation and progression of breast cancer as indicated by being the cause of the Cowden's breast cancer predisposition syndrome, being mutated in a subset of breast cancers, particularly advanced breast cancer and by being decreased in breast cancers by epigenetic mechanism. Recent evidence suggests that decreased PTEN levels or function rather than complete deletion may contribute to cancer development. Our preliminary date with breast cancer cells lacking MMAC1 have demonstrated that reintroduction of MMAC1 results in cell cycle arrest of apoptosis dependent on whether cells have additional viability signals provided by serum or by ligation of integrins. Deletion of MMAC 1 in knockout mice results in lethality in the homozygous state and tumor development in multiple organs in a heterozygous state. Attempts to develop tissue specific knock outs of MMAC1 have failed to provide useful models due to leakiness and tumor development in other organs. To provide a model that mimics the situation in human breast cancer, we will establish tissue specific transgenic murine models with increased and decreased MMAC1 function by expression of wild type or a dominant negative MMAC1.
We have 1) generated trangene constructs for wild type MMAC1 under the MMTV promoter, as dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter, a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact and a WAP MMAC1 dominant negative (c/s). 2) established transgenic mice and demonstrated breast specific expression of wild type MMAC1 under the MMTV promoter (1 founder), a dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter (3 founders) a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact (7 founders), and a WAP MMAC1 dominant negative (1 founder). 3) followed each strain of mice with at least 15 progeny for 6 months to establish sensitivity to tumor development. 4) collected tissues to determine effect on breast development, and 5) transferred each transgenic strain to the C57/B16 background by breeding and are at the F1-F4 generation.
We will assess tumor and breast development in each of the transgenic lines.
NOTE: Two graduate students, Muling Mao and Bruce Cuevas have graduated with Ph.D.s based on this support.

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Introduction

MMAC1/PTEN/TEP1 is a tumor suppressor gene on 10q23, which is aberrant at the level of Loss of heterozygosity (LOH), mutation, methylation, protein stability and function in a significant portion of breast cancers (1-4). Germ line mutations in MMAC1 are responsible for the Cowden's breast cancer predisposition syndrome, validating MMAC1 as a critical target in breast tumorigenesis (5,6). As reported, about 30% of women from the Cowden disease families developed cancer, of which the great majority (78%) was of the breast. MMAC1 is also functionally inactivated in a high proportion of leukemia and lymphomas by methylation independent mechanisms (7). Therefore, MMAC1 is an important tumor suppressor gene, particularly in breast tissue.

LOH of MMAC1 is a frequent (>40%) event in invasive breast cancers (3,4). In support of the concept of functional inactivation of MMAC1 in breast cancer, 15% of invasive ductal breast cancers lack detectable MMAC1 protein and 18% have decreased MMAC1 protein levels with the majority of cases demonstrating LOH at 10q23. Inactivation of MMAC1 may represent a late event, as MMAC1 mutations occur at a higher frequency in advanced breast cancers and LOH at 10q23 is strongly correlated with increased tumor invasiveness and poor differentiation (3,4). The stability and enzyme activity of the MMAC1 protein are regulated by phosphorylation at serine, and, as we have demonstrated, tyrosine residues by growth factors and src tyrosine kinases (8,9). Thus, the function of MMAC1 and its down stream pathway may be altered in breast cancers by post-transcriptional modification as well as by genetic effects.

MMAC1 dephosphorylates the site on membrane phosphatidylinositols (PtdIns) phosphorylated by phosphatidylinositol 3 kinase (PI3K) (10,11). MMAC1 also dephosphorylates serine, threonine and tyrosine residues in proteins (12). Thus, MMAC1 has the ability to dephosphorylate proteins, lipids and inositols, suggesting that MMAC1 may alter the activity of multiple proteins as well as of the PtdIns pathway. Introduction of a wild-type MMAC1 gene into breast, prostate, or glioma cancer cell lines lacking functional MMAC1 protein decreases signaling through the PI3K pathway as indicated by a decrease in PtdIns3,4,5 P3 levels and alterations in downstream events including phosphorylation or localization of AKT, p70S6K, GSK3-α, GSK3-β, Bad, and 4E-BP1 (13-16).

The PI3K target, AKT is the cellular homologue of the v-AKT oncogene and has been widely shown to be involved in survival signals induced by various growth factors (17, 18). The pleckstrin

homology (PH) domain on AKT mediates high affinity interactions of AKT with the PI3K lipid product, PtdIns (3,5) P2 resulting in AKT recruitment to the cell membrane for activation (19). Full activation of AKT is at least partially dependent on phosphorylation of thr 308 and ser 473 by PDK1 and PDK2 respectively (20). Activated AKT is thought to mediate the activation of cell cycle mediators and inactivation of apoptotic proteins.

Developing transgenic mice, breeding, and evaluation is a time consuming process that allows for additional research to be performed while waiting for the mice to develop tumors. Two graduate students, Bruce Cuevas and Muling Mao were supported by this grant. Both have graduated with PhDs based on studies of the functional regulation of the MMAC1 pathway.

Body

Task #1 To develop and establish transgenic mouse models which display mammary gland specific expression of the wildtype and inactive forms of the MMAC1 tumor suppressor.

We have inserted the wild type (wt) MMAC1 gene into murine mammary tumor virus (MMTV) vector that specifically targets mammary tissue. An inactive form of MMAC1, which has the conserved Cys124 at the phosphatase domain changed to serine (c/s), was also inserted downstream of MMTV promotor. This construct inhibits both the lipid and protein phosphatase activity of MMAC1. In addition, we prepared a whey acidic protein (WAP) construct containing the c/s MMAC1 construct. The WAP promotor also targets to breast, but compared with MMTV regulates adequate gene expression during mid-pregnancy. Moreover, a Gly129Glu (g/e) phosphoinositide phosphotase-deficient MMAC1 was cloned into MMTV promotor. MMAC1 (g/e) lacks the ability to dephosphorylate lipids while leaving the protein phosphotase activity intact thus selectively acting as a dominant negative for the lipid phosphatase activity of MMAC1.

Task #2 To determine the effects of MMAC1 expression on mammary development and tumorigenesis.

We have obtained founders from each transgenic line: one founder for MMTV wt, three founders for MMTV c/s, one founder for WAP c/s and seven founders for MMTVG/E. Typical examples of positive mice in terms of expression assessed by cutting with BamH1 and HindII and southern blotting or PCR with primers unique to the transgene are presented in figure 1. To distinguish the heterozygous from non-transgenic mice, tail DNA was digested, separated on and agarose gel and hybridized with a ^{32}P-labeled MMAC1 cDNA probe. For a more sensitive approach, mouse tail DNA was amplified with PCR primers derived from MMAC1 cDNA and a transgene specific primer. The PCR products were examined on an agarose gel with the transgene status indicated by the band above the primer dimers. Expression of MMAC1 protein in the transgenic mice was determined by western blot with anti-MMAC1 antibody of breast tissue (Figure 1). The results indicated variable expression levels of the transgenes among the lines. Equal loading of samples was evaluated with blotting the membrane with anti-β-actin antibody.

Mice with relatively high expression of transgenes have been bred and followed for at least 6 months. We have followed 15 MMTV wt, 14 MMTV c/s, 35 MMTVg/e mice and 15 WAP c/s

mice. None of the mice have yet developed palpable tumors. The mice will be followed for an additional year. A similar number of mice will be bred multiple times to assess the effects of pregnancy and the resultant high level expression of the transgene on tumor development.

We have collected tissues from the mice to determine the effects of the transgene on breast development. These samples will be assessed with the assistance of Rhama Kohkhar a breast pathologist with experience in murine models. The mice will be bred to determine the effects on lactation and breast involution.

Task #3 To determine the effects of MMAC1 on breast tumorigenesis induced by other oncogenes and tumor suppressor genes.

To allow assessment of MMAC1 wt and dn constructs on tumor development, we need to cross the mice into a background where the appropriate transgenes or knockouts are available. We have crossed the lines with C57/Bl6 mice and are at the F4 generation.

In the event that the MMAC1 transgenic mice do not produce primary tumors following the manipulations described above, we will cross thee mice with Wnt1 transgenic or p27 knock-out mice which are commercially available. Wnt1 and p27 have been shown to complement PTEN in tumor development in breast and prostate models. We will also assess the mice in a polyoma middle T system developed by Dr. W. Muller. Polyoma middle T is a potent tumor inducer. However, a mutant polyoma middle T which is incapable of linking to the phosphatidylinositol 3 kinase pathway does not induce tumors. We will assess whether activation or inhibition of MMAC1 function using the transgenes available will alter tumor development in the wild type and mutant polyoma system.

Developing transgenic mice, breeding, and evaluation is a time consuming process that allows for additional research to be performed while waiting for the mice to develop tumors. Two graduate students, Bruce Cuevas and Muling Mao were supported by this grant. Both have graduated with PhDs based on studies of the functional regulation of the MMAC1 pathway. These studies are described in the following papers (Appended).

Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K.A., and Mills G.B., 1999 SHP-1 regulates LCK-induced phosphatidylinositol 3' kinase phosphorylation and activity. J. Biol Chem: 274:27583-27589.

Cuevas, B.D., Lu. Y., Mao, M., Zhang, J., Lapushin R., Siminovitch, K., and Mills G.B. 2001 Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. J. Biol Chem. 276:27455-27461.

Mao M., Fang X.J., Lu, Y., Lapushin R., Bast R.C., and Mills G.B. 2000 Inhibition of growth factor-induced phosphorylation and activation of PKB/AKT by atypical PKCz in breast cancer cells Biochem J. 352:475-482.

Mao M., Cuevas, B., Lu, Y., Wang, H., and Mills, G.B., 2003 Association and Regulation of PKCδ on PI3K by Phorbol Esters and Oxidants through Src kinases Submitted

Key Research Accomplishments

1). Generated trangene constructs for wild type MMAC1 under the MMTV promoter, a dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter, a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact and a WAP MMAC1 dominant negative (c/s).

2) established transgenic mice and demonstrated breast specific expression of wild type MMAC1 under the MMTV promoter (1 founder), a dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter (3 founders) a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact (7 founders), and a WAP MMAC1 dominant negative (1 founder).

3) followed each strain of mice with at least 15 progeny for 6 months to establish sensitivity to tumor development.

4) collected tissues to determine effect on breast development

5). Transferred each transgenic strain to the C57/Bl6 background by breeding and are at the F1-F4 generation.

6) Graduated 2 students with PhDs. Bruce Cuevas, Muling Mao

Reportable Outcomes

Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K.A., and Mills G.B., 1999 SHP-1 regulates LCK-induced phosphatidylinositol 3' kinase phosphorylation and activity. J. Biol Chem: 274:27583-27589.

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Muling Mao and Bruce Cuevas have both graduated with PhDs from the University of Texas.

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Appendices

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SHP-1 Regulates Lck-induced Phosphatidylinositol 3-Kinase Phosphorylation and Activity*

(Received for publication, January 21, 1999, and in revised form, June 1, 1999)

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Ligation of the T cell antigen receptor (TCR) activates the Src family tyrosine kinase p56 Lck, which, in turn, phosphorylates a variety of intracellular substrates. The phosphatidylinositol 3-kinase (PI3K) and the tyrosine phosphatase SHP-1 are two Lck substrates that have been implicated in TCR signaling. In this study, we demonstrate that SHP-1 co-immunoprecipitates with the p85 regulatory subunit of PI3K in Jurkat T cells, and that this association is increased by ligation of the TCR complex. Co-expression of SHP-1 and PI3K with a constitutively activated form of Lck in COS7 cells demonstrated the carboxyl-terminal SH2 domain of PI3K to inducibly associate with the full-length SHP-1 protein. By contrast, a truncated SHP-1 mutant lacking the Lck phosphorylation site (Tyr^{564}) failed to bind p85. Wild-type but not catalytically inactive SHP-1 induced dephosphorylation of p85. Furthermore, expression of SHP-1 decreased PI3K enzyme activity in anti-phosphotyrosine immunoprecipitates and phosphorylation of serine 473 in Akt, a process dependent on PI3K activity. These results indicate the presence of a functional interaction between PI3K and SHP-1 and suggest that PI3K signaling, which has been implicated in cell proliferation, apoptosis, cytoskeletal reorganization, and many other biological activities, can be regulated by SHP-1 in T lymphocytes.

In the context of appropriate co-stimulatory signals, ligation of the T cell antigen receptor (TCR)[1] by antigenic peptide bound to a major histocompatibility complex molecule leads to T cell activation and ultimately, a functional immune response. Activation of protein tyrosine kinases and consequent intracellular protein phosphorylation are among the first events elicited by TCR ligation and are crucial to the induction of biochemical pathways that regulate cell growth (1). This protein-tyrosine kinase activity, together with opposing protein-tyrosine phosphatase activity, plays a major role in regulating the magnitude of TCR-induced tyrosine phosphorylation, as well as the duration and termination of cell activation (1, 2). The counterbalance of tyrosine kinases by tyrosine phosphatases is integral to the maintenance of cellular homeostasis (3, 4), and disruption of this balance has been shown to be a hallmark of cellular transformation (5).

P56 Lck is a member of the Src family of non-receptor tyrosine kinases which is highly expressed in T lymphocytes (6). Along with the Fyn Src family kinase and the ζ-associated protein 70 (ZAP-70), Lck has been implicated in the initial activation events resulting from TCR ligation (1, 2, 6). Lck has been shown to associate with the CD4 and CD8 T cell surface antigens (6), and to play an integral role in the ligand-induced phosphorylation of the TCR intracellular components (1, 2, 6). Indeed, Lck-mediated phosphorylation of the ζ subunit of the TCR and ZAP-70 couples TCR ligation to a variety of downstream signaling molecules (2), and the loss of Lck activity significantly reduces the capacity of the TCR to transduce activation signals (7).

SHP-1 is an SH2 domain-containing non-receptor tyrosine phosphatase implicated in the negative regulation of a number of growth factor receptors, including the B and T cell antigen, erythropoeitin, the platelet-derived growth factor (PDGFR), c-kit, and the granulocyte macrophage colony-stimulating factor receptors (8–13). SHP-1 is highly expressed in T cells (4), and has also been linked to the negative regulation of TCR signaling (14–16). This effect of SHP-1 appears to reflect its capacity to down-regulate ZAP-70 (14) and Lck (17) activities and to also dephosphorylate TCR components and downstream signaling molecules (15, 16). SHP-1 has been shown to undergo tyrosine phosphorylation in response to CD4 or CD8 stimulation as well as Lck activation (18). As is consistent with an inhibitory effect of SHP-1 on TCR signaling, thymocytes from SHP-1-deficient viable motheaten exhibit a significantly increased proliferative response to stimulation by anti-CD3 antibodies as compared with normal mouse thymocytes (16, 17).

Ligation of the TCR alters inositol lipid metabolism through induction of phosphatidylinositol 3′-kinase (PI3K) activity (1). PI3K consists of a p85 regulatory subunit with two SH2 domains and a SH3 domain, and a p110 catalytic subunit which phosphorylates the 3′-hydroxyl of the inositol ring of phosphatidylinositol (19, 20). The resulting PI3K products bind to pleckstrin homology (PH) domains of intracellular signaling molecules recruiting them to the cell membrane. Activation of the PH domain containing c-Akt (21, 22) has been associated with cell cycle progression (23, 24) and the propagation of an anti-apoptotic signal (22, 25–27). Jurkat T cell activation via anti-CD3 antibody binding to the TCR complex has been shown to result in the rapid phosphorylation of both PI3K subunits

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[1] The abbreviations used are: TCR, T cell antigen receptor; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; HAp85, hemagglutinin epitope tag-labeled p85 construct; SH2, Src homology domain 2.

(28), as well as an accumulation of PI3K products (29). TCR-induced tyrosine phosphorylation of Tyr^{688} in the p85 subunit of PI3K and the consequent activation of PI3K have been linked to the presence of Lck (28, 30), and other recent data provide additional evidence of a role for Lck in PI3K signaling (31). However, the phosphatase(s) that dephosphorylates PI3K has not been identified as of yet.

In this study, we demonstrate that Lck activity is associated with an interaction of SHP-1 with the p85 subunit of PI3K, and also identify p85 as a target for SHP-1-mediated dephosphorylation. The association between p85 and SHP-1 requires tyrosine phosphorylation of SHP-1 and likely involves binding of SHP-1 phosphotyrosine 564 to the p85 carboxyl-terminal SH2 domain via a novel tyrosine recognition motif. This interaction is also associated with a reduction in the lipid kinase activity in total anti-phosphotyrosine immunoprecipitates and a reduction in PI3K-mediated phosphorylation of Akt. Together, these findings implicate the interaction of SHP-1 with PI3K in the modulation of the PI3K signaling cascade downstream of TCR engagement.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—A monoclonal antibody against the ε chain of human CD3 complex (UCHT1, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Peter Beverly (University College, London, United Kingdom). The rabbit polyclonal antibody against Lck was described previously (32). The anti-phosphotyrosine monoclonal antibody (4G10, IgG1) and the rabbit polyclonal antibody against the p85 subunit of PI3K, and the rabbit polyclonal antibody against SHP-1 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against Akt and phospho-Akt were purchased from New England Biolabs (Beverly, MA). A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas at Houston). Rabbit anti-mouse IgG was purchased from Western Blotting Inc. (Toronto, ON). Horseradish peroxidase goat anti-mouse IgG was purchased from Bio-Rad. Glutathione-Sepharose and protein A-Sepharose beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GST fusion proteins of the p85 SH2 domains were generous gifts of Dr. T. Pawson (Toronto, ON). The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Veillette (Montreal, QE). The cDNA plasmid for HAAkt was a generous gift of Dr. Julian Downward (London, United Kingdom). The cDNA plasmid for HAp85 and ΔHAp85 were described previously (33).

Cell Lines—Human Leukemic Jurkat T cell line E6.1, and COS7 cells were purchased from American Type Culture Collection (Rockville, MD).

Cell Culture, Stimulation, and Lysis—Jurkat T and COS7 cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) containing penicillin/streptomycin (1%, Life Technologies, Inc.), L-glutamine (2 mM, Life Technologies, Inc.), and 10% (v/v) fetal calf serum (Sigma) at 37 °C in a humidified atmosphere. For CD3 cross-linking, cells were incubated with anti-CD3 (0.6 μg/ml) antibodies plus rabbit anti-mouse IgG (10 μg/ml) at room temperature for the indicated time periods. After stimulation, the cells were pelleted, resuspended in 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.4, 1 mM sodium orthovanadate, 50 mM $ZnCl_2$, 50 mM sodium fluoride, 50 mM sodium orthophosphate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and incubated at 4 °C for 20 min. After centrifugation at 14,000 × *g* for 5 min at 4 °C, post-nuclear detergent cell lysates were collected.

Transient Transfection—COS7 cells were transfected by Lipofection. Briefly, 4 × 10^5 cells were seeded on 100-mm cell culture plates and incubated in complete media overnight. cDNA expression constructs were incubated in serum-free medium with LipofectAMINE (Life Technologies, Inc.) at room temperature for 30 min, then diluted with serum-free medium and incubated with cells at 37 °C for 2 h, after which time the LipofectAMINE mixture was replaced with complete media and the cells were returned to 37 °C for 24 h. Complete media was then removed, the cells rinsed, and incubation continued with serum-free medium for an additional 24 h.

Immunoprecipitation and Immunoblotting—Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4 °C for 2 h followed by another 2-h incubation with protein A-Sepharose beads. The immunoprecipitates were washed with IP wash buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), anti-phosphotyrosine (1:3000), or anti-SHP-1 (1:400) at room temperature for 2 h. Horseradish peroxidase-protein A or horseradish peroxidase-goat anti-mouse IgG was used as the secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL, Amersham). Where indicated, blots were stripped by treatment with 2% SDS and 100 mM β-mercaptoethanol in Tris-buffered saline and then reprobed with anti-p85 PI3K antibodies and detected by ECL.

Fusion Protein Binding Assays—Transfected COS7 cells were starved for 24 h in serum-free medium. The cells were lysed in Nonidet P-40 lysis buffer. Bacterial lysates containing the fusion protein GST alone, the p85 amino-terminal SH2 domain, or the p85 carboxyl-terminal SH2 domain were diluted in phosphate-buffered saline and incubated with glutathione-Sepharose beads. GST fusion protein beads were washed, then incubated with the transfected cell lysate at 4 °C for 2 h. After extensive washing, the proteins were eluted and immunoblotted as described above.

Kinase Activity—Cells were lysed in 1% Nonidet P-40 lysis buffer. Cell lysates normalized for protein levels (BCA assay; Pierce Chemical Co., Rockford, IL) were immunoprecipitated using anti-HA and protein A-Sepharose. Non-transfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described (34). Briefly, the immunoprecipitates were washed sequentially in: (*a*) phosphate-buffered saline, 100 μM Na_3VO_4, 1% Triton X-100; (*b*) 100 mM Tris, pH 7.6, 0.5 M LiCl, 100 μM Na_3VO_4; (*c*) 100 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 100 μM Na_3VO_4; (*d*) 20 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 30 mM $NaPP_i$, 200 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.03% Triton X-100, and resuspended in 30 μl of kinase reaction buffer (33 μM Tris, pH 7.6, 125 mM NaCl, 15 μM $MgCl_2$, 200 μM adenosine, 15 μM ATP, 30 μCi of [γ-^{32}P]ATP). Phosphatidylinositol (PI) was resuspended in 20 mM Hepes, pH 7.5, at 2 mg/ml and sonicated on ice for 10 min. The PI 3-kinase reaction was initiated by adding 10 μl of the PI suspension. The reaction proceeded for 30 min at room temperature and was terminated by adding 100 μl of 1 N HCl. Lipids were extracted by 600 μl of chloroform:methanol (1:1). The organic phase was washed with H_2O, collected and dried by vacuum centifugation. The lipids were resuspended in 20 μl of chloroform:methanol (1:1) and resolved on Silica Gel G-60 thin-layer chromatography (TLC) plates in cloroform:methanol:NH_4OH:H_2O (60:47:2:11.3). Radiolabeled phosphatidylinositol phosphate was visualized by autoradiography.

Lck Autophosphorylation Assay—Cells were lysed in kinase lysis buffer (35). Cell lysates normalized for protein levels were immunoprecipitated using a rabbit antibody against human Lck and protein A-Sepharose. Non-transfected COS7 and SHP-1 transfected cell lysates were used as negative controls. After immunoprecipitation, the beads were washed four times with wash buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Hepes, pH 7.5, 1 mM Na_3VO_4). The washed beads are then resuspended in 50 μl of kinase reaction mixture (20 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 μM ATP, 10 μCi of [γ-^{32}P]ATP) and incubated at room temperature for 30 min. The reaction was stopped by washing the beads twice with wash buffer including 1 mM EDTA. Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Radiolabeled Lck was visualized by autoradiography.

Subcellular Fractionation—Jurkat cells were incubated in serum-free RPMI for 16 h prior to stimulation. Cells were divided into two aliquots (25 × 10^6 cell each), and one was stimulated by cross-linking TCR complex proteins with anti-CD3 (see above) for 7 min. Membrane and cytosolic fractions were separated based on the protocol of Resh and Erickson (36). Briefly, cells were washed twice with STE (150 mM NaCl, 50 mM Tris, 1 mM EDTA) and collected with low speed centrifugation (1,000 × *g*). The cells were resuspended in hypotonic lysis buffer (10 mM Tris, 0.2 mM $MgCl_2$, 5 mM KCl, 1 mM $NaVO_4$, pH 7.4) and incubated on ice for 15 min. The cells were lysed with 30 strokes in a Dounce homogenizer. Lysates were adjusted to 0.25 M sucrose, 1 mM EDTA, and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatant was removed, and the pellet resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and given five additional strokes in a Dounce homogenizer, and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatants were combined and centifuged at 100,000 × *g* for 1 h. The result-

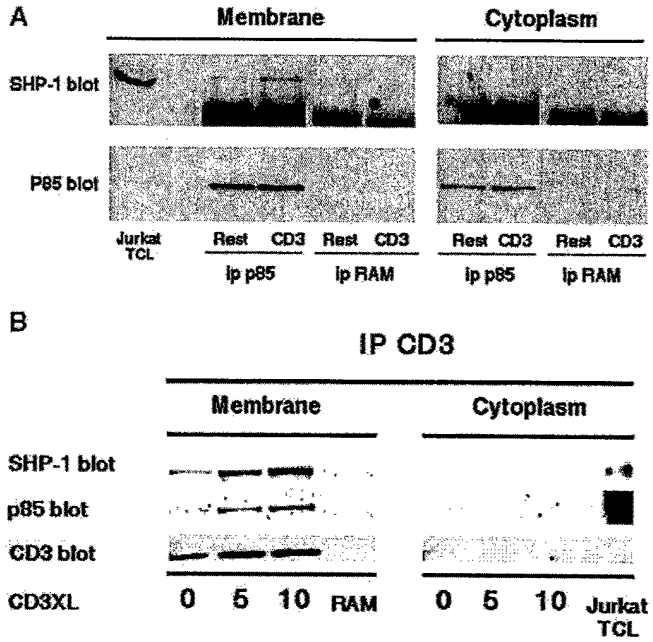

FIG. 1. **SHP-1 co-immunoprecipitates with p85 regulatory subunit in Jurkat cells.** Jurkat T cells were stimulated with anti-CD3 antibodies, lysed, and subjected to subcellular fractionation. *A*, equivalent amounts of protein from resting and stimulated fractions were immunoprecipitated with antibody to p85 and the precipitated proteins then subjected to immunoblotting with anti-SHP-1 antibody. The p85 immunoblot (*lower panel*) demonstrates equal loading of the test samples. An identical set of samples was immunoprecipitated (*ip*) with rabbit anti-mouse (*RAM*) antibody as a control. Data are representative of three independent experiments. *B*, membrane fraction was immunoprecipitated with anti-CD3 antibody and the precipitated proteins subjected to immunoblotting with anti-SHP-1 and anti-p85 antibodies, with an anti-CD3 immunoblot performed to demonstrate equal loading.

ing supernatant was labeled S100 (cytosolic), and the pellet labeled P100 (membrane). The P100 fraction was resuspended in phosphate-buffered saline. All samples were pre-cleared with protein A-Sepharose for 1 h at 4 °C. Both fractions were divided into two samples each, with one sample to be immunoprecipitated with anti-p85 antibody, and the other with rabbit anti-mouse antibody as a negative control.

RESULTS

SHP-1 Physically Associates with PI3K—Although PI3K has been shown to be phosphorylated and activated following TCR ligation (28), the phosphatase responsible for dephosphorylation of PI3K has yet to be identified. The tyrosine phosphatase SHP-1 has been shown to target a number of molecules required for TCR signal relay (4). To address the possibility that PI3K represents a SHP-1 target, the capacity for SHP-1 to associate with PI3K in TCR-stimulated Jurkat cells was investigated by cross-linking the TCR complex with antibodies to CD3. We utilized a subcellular fractionation approach (36) to maximize the yield of activated, membrane-associated PI3K and reduce dilution by non-activated PI3K. Results of immunoblotting analysis indicated SHP-1 to be present in p85 immunoprecipitates from the membrane fraction (Fig. 1*A*, representative of three experiments) but not the cytosolic fractions of Jurkat T cells. TCR ligation resulted in a doubling, as assessed by densitometric analysis, of the amount of SHP-1 associated with p85 (Fig. 1, *lanes 1* and *2*), a result which is suggestive of recruitment of SHP-1 to a complex containing PI3K upon activation. Compatible with the presence of SHP-1 in PI3K immunoprecipitates, CD3 ligation induced a time-dependent increase in the amount of SHP-1 and PI3K present in membrane fraction anti-CD3 immunoprecipitates (Fig. 1*B*). The similar kinetics of association of SHP-1 and p85 with the TCR place these two signaling proteins at the activated TCR at the same time, and provide further evidence of a complex containing both SHP-1 and PI3K. Thus SHP-1, both constitutively and inducibly, associates with membrane bound and presumably activated PI3K in Jurkat cells (19, 20, 37, 38), either directly or as part of a multimeric complex. Whether the baseline association of these proteins reflects constitutive activation of Jurkat cells, even in serum-free medium, remains to be determined.

The p85 Carboxyl-terminal SH2 Domain Binds Phosphorylated SHP-1—To determine the functional relationship between PI3K and SHP-1, we used a transient transfection system involving the expression of recombinant p85 and SHP-1 in COS7 cells. T cell receptor activation was simulated in this system by overexpression of a constitutively activated form of Lck (Lck Y505F) that was generated by mutating the regulatory carboxyl-terminal inhibitory tyrosine (6). In previous studies, the regulatory PI3K subunit p85 has been shown to be phosphorylated by Lck Y505F when these proteins are co-expressed in COS1 cells (30). The major site of Lck-induced p85 phosphorylation has been mapped to a tyrosine residue (Tyr^{688}) located within the carboxyl-terminal SH2 domain (30). As Tyr^{564} in the SHP-1 carboxyl-terminal tail is also phosphorylated by Lck, and both p85 and SHP-1 contain SH2 domains, Lck-induced physical association of p85 with SHP-1 might be mediated by binding of the p85 SH2 domain(s) to phosphotyrosine on SHP-1. Alternatively, the SH2 domain of SHP-1 might inducibly associate with phosphorylated p85. To distinguish between these possibilities, the capacity of GST fusion proteins containing the p85 amino- or carboxyl-terminal SH2 domains to precipitate SHP-1 from lysates of transfected COS7 cells was examined. For these studies, the cells were transfected with a catalytically inactive form of SHP-1 (SHP-1 C453S) so as to prevent autodephosphorylation (18) and thus maximize the level of SHP-1 phosphorylation. As illustrated by the anti-SHP-1 Western blot shown in Fig. 2*A*, the results of this analysis revealed only the carboxyl-terminal SH2 domain of p85 to bind SHP-1 C453S, and indicated this association to require the presence of Lck Y505F. By contrast, tyrosine-phosphorylated p85 was not precipitated by GST-SHP-1 SH2 domain fusion proteins (data not shown). To determine whether the major site on SHP-1 for Lck-mediated phosphorylation (18) was involved in the p85 SH2-mediated association between p85 and SHP-1, a truncation mutant construct (SHP-1 Δ35) encoding amino acids 1 through Lys^{560} of SHP-1 and thus lacking Tyr^{564}, was derived and its capacity to associate with the p85 carboxyl-terminal SH2 domain then examined in the transfected COS7 cells. As illustrated by Fig. 2*B*, immunoblot analysis revealed the failure of SHP-1 Δ35 to associate with the p85 carboxyl-terminal domain, and thus demonstrated this association to require one or more amino acids mapping within the Δ35 segment. As Tyr^{564}, located within the last 35 amino acids of SHP-1, is the primary site of Lck phosphorylation in SHP-1, and Lck is required for the association of SHP-1 with the carboxyl-terminal SH2 domain of PI3K (Fig. 2*A*), these data strongly suggest that it is the interaction of this phosphorylated residue with the p85 carboxyl-terminal SH2 domain which mediates physical association of p85 with SHP-1.

SHP-1 dephosphorylates Lck-phosphorylated p85—Association of the p85 SH2 domain with the carboxyl terminus of SHP-1 creates the opportunity for SHP-1 to dephosphorylate Tyr^{688} of p85 (Fig. 2*C*), the major site of Lck phosphorylation on p85 (30). Accordingly, the possibility that SHP-1 dephosphorylates Lck-phosphorylated p85 was investigated in COS7 cells co-transfected with a recombinant hemagglutinin epitope tag-labeled p85 construct (HAp85), Lck Y505F, and SHP-1. Immunoprecipitation of HAp85, followed by SDS-PAGE and Western blotting with anti-phosphotyrosine clearly demonstrate the co-

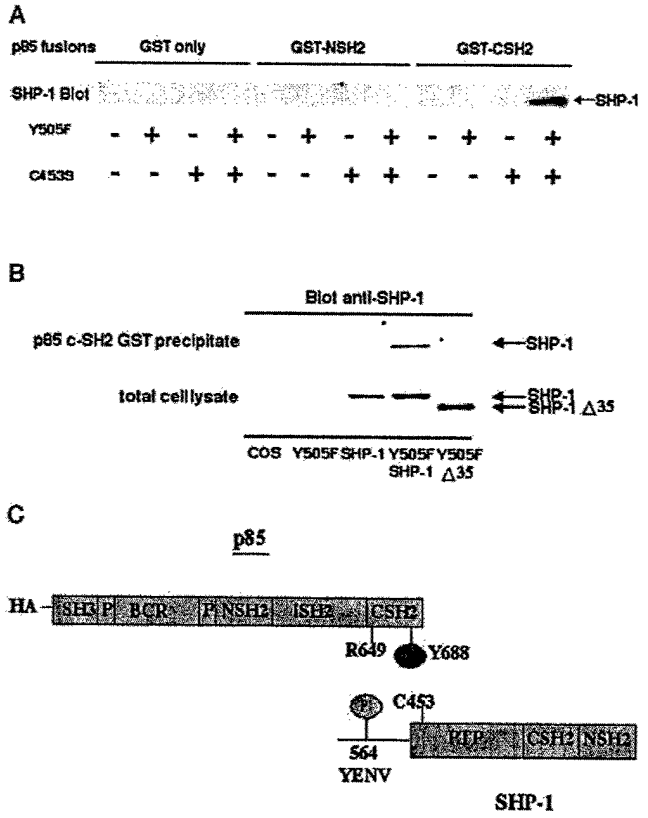

FIG. 2. **The p85 carboxyl-terminal SH2 domain binds phosphorylated SHP-1.** COS7 cells were transfected, then expanded in culture for 48 h prior to cell lysis. *A*, COS7 cells were transfected with SHP-1 C453S, Lck Y505F, or with both SHP-1 C453S and Lck Y505F. Lysates were mixed with either GST alone or GST-p85 SH2 fusion proteins immobilized on glutathione-agarose beads. Bound proteins were separated by SDS-PAGE and transferred to an Immobilon membrane. Bound SHP-1 C453S was detected by probing the membrane with anti-SHP-1. *B*, SHP-1 Δ35 transfected COS7 cell lysate is included as a test sample in a repeat of the GST-p85 carboxyl-terminal SH2 fusion protein binding assay. Data are representative of three independent experiments. *C*, a schematic depicting the proposed model of SHP-1 association with p85 is shown.

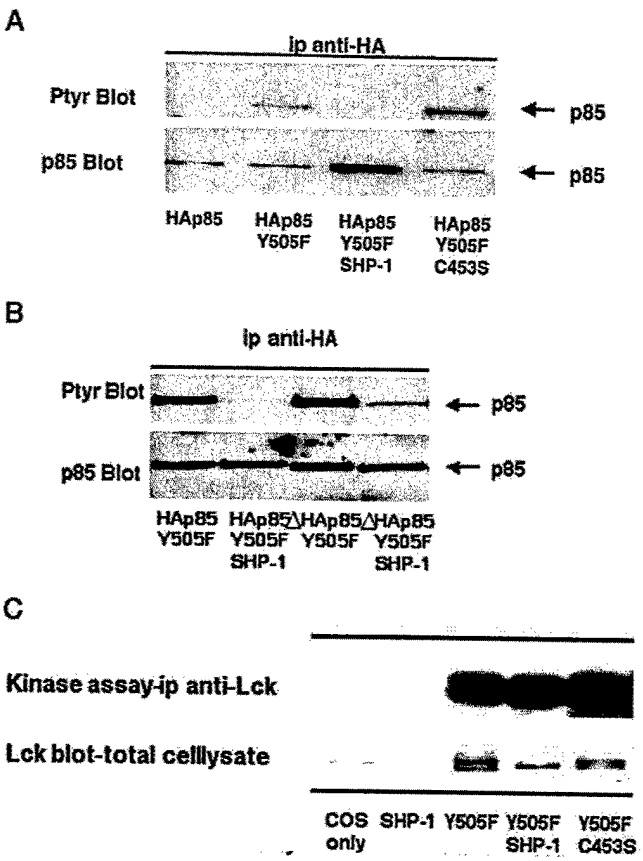

FIG. 3. **SHP-1 dephosphorylates p85.** *A* and *B*, COS7 cells were transfected as indicated. HA epitope-tagged p85 was immunoprecipitated with anti-HA antibodies, and the proteins separated by SDS-PAGE followed by transfer to Immobilon. Tyrosine-phosphorylated p85 was detected by probing with anti-phosphotyrosine antibodies. Data are representative of four independent experiments. *C*, COS7 cells were transfected with Lck Y505F and SHP-1 or SHP-1 C453S, and lysed after 48 h. The lysates were immunoprecipitated with anti-Lck antibody and analyzed by Lck autokinase assay. Data are representative of three independent experiments.

transfection of HAp85 with Y505F to induce a level of tyrosine phosphorylation of the recombinant p85 protein which is significantly increased relative to the vector control (Fig. 3*A*, *lanes 1* and *2*). Expression of SHP-1 with Y505F and HAp85 in this system was associated with a reduction of p85 phosphorylation to a level comparable to that detected in vector control cells (Fig. 3*A*, *lanes 1* and *3*). Thus p85 appears to represent a SHP-1 substrate. Interestingly, substitution of wild-type SHP-1 with SHP-1 C453S not only restored p85 phosphorylation to the level detected in the Y505F/HAp85 lysate, but also engendered the highest p85 phosphorylation detected in any transfectant (Fig. 3*A*).

As p85 heterodimerizes with the p110 subunit of PI3K, the possibility that association with p110 was required for SHP-1-mediated dephosphorylation of p85 was also studied. To this end, the Lck Y505F transfected COS7 cells were also co-transfected with a mutant form of p85 (ΔHAp85) (39) in which the inter-SH2 (iSH2) p110-binding region, that is absolutely required for p85 heterodimerization (Fig. 2*C*), was deleted. Analysis of these cells revealed ΔHAp85 to be both phosphorylated by activated Lck, and dephosphorylated by SHP-1 (Fig. 3*B*). Thus, while the physical association between p110 and SHP-1 cannot be excluded, these data suggest that such an association is not necessary for the SHP-1-mediated dephosphorylation of p85.

Although the Lck Y505F mutant used in these studies lacks the regulatory carboxyl tyrosine, it is possible that the effects of SHP-1 on p85 phosphorylation relate to SHP-1-mediated dephosphorylation of other phosphotyrosine sites in Lck and consequent down-regulation of Lck Y505F activity. To assess this possibility, Y505F autophosphorylation *in vitro* was examined in COS7 cells transfected with Lck Y505F alone or in combination with either SHP-1 or SHP-1 C453S. The results of this assay revealed the *in vitro* kinase activity of Lck Y505F to remain intact in the presence of SHP-1 expression (Fig. 3*C*). Taken together, these data indicate that p85 not only physically associates with SHP-1, but also is dephosphorylated by SHP-1.

Effect of SHP-1 Expression on PI3K Activity—To determine whether SHP-1-mediated dephosphorylation of p85 is associated with a change in PI3K activity, epitope-tagged p85 was immunoprecipitated from COS7 co-transfectants and the kinase activity of the associated p110 catalytic subunit was evaluated using an *in vitro* lipid phosphorylation assay. The results of this analysis revealed PI3K lipid kinase activity to be unaffected by SHP-1 expression (data not shown). However, as SHP-1 interaction with PI3K involves PI3K tyrosine phosphorylation, the possibility that SHP-1 binding diminishes activity of phosphorylated, but not total cellular PI3K, was also addressed. To this end, anti-phosphotyrosine antibodies were used to immunoprecipitate phosphorylated proteins from the COS7 lysates, and the precipitated phosphoproteins were then

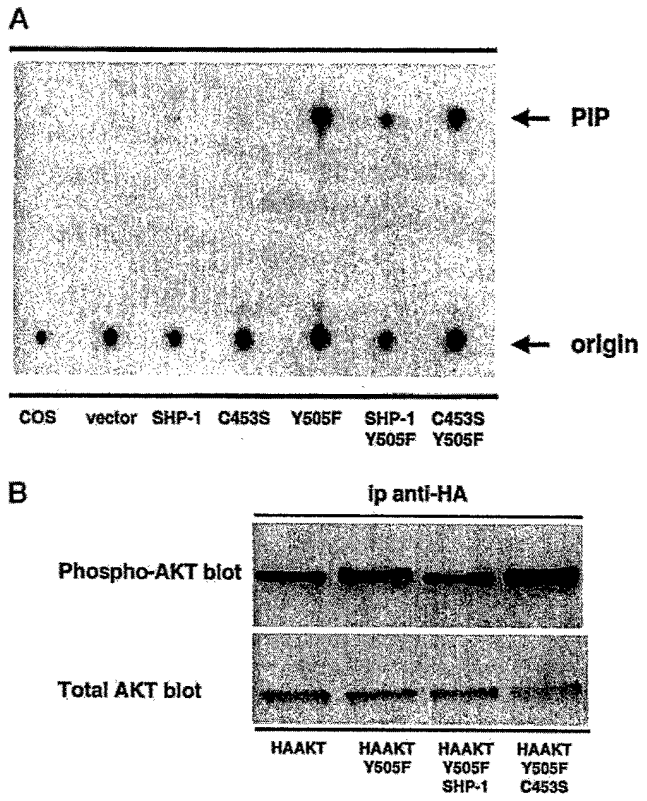

FIG. 4. **SHP-1 expression results in a decrease of PI3K activity.** *A*, COS7 cells were transfected as indicated previously. Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies, and then subjected to an *in vitro* lipid kinase assay. The assay mixture was separated on thin-layer chromatography plates, and 3'-phosphorylated lipids detected by autoradiography. *PIP*, phosphatidylinositol. *B*, anti-HA immunoprecipitates (*ip*) were separated and transferred to Immobilon and the filters probed with antibody to Ser^{473}-phosphorylated AKT. A subsequent immunoblot with antibody to total AKT was performed to demonstrate equal loading. *C* represents the densitometric variation of the co-transfected samples in *B* as compared with the sample transfected with HAAkt. HAAkt is arbitrarily set as 100. Data are representative of four independent experiments.

evaluated for lipid kinase activity. Results of this analysis revealed the lipid kinase activity present in the tyrosine-phosphorylated fraction to be markedly reduced in the Lck Y505F/SHP-1 co-transfectants as compared with the transfectants in which Lck Y505F was expressed in the absence of SHP-1 (Fig. 4*A*). By contrast, expression of SHP-1 C453S did not affect anti-phosphotyrosine immunoprecipitable lipid kinase activity, a result which indicates the decreased PI3K activity observed in the Lck Y505F/SHP-1 cells to be dependent on the phosphatase activity of SHP-1.

The regulatory effects of SHP-1 on PI3K signaling were also investigated by analyzing the relevance of SHP-1 to the activities of signaling molecules downstream of PI3K. Most notable among the latter proteins is Akt, a PH domain-containing kinase linked to cell cycle progression, proliferation, and cell death (40). Phosphorylation of Akt at serine residue 473 (S473) is absolutely dependent on PI3K activity (22), being abrogated by PI3K inhibitors LY294002 and wortmannin (data not shown). Evaluation of PI3K-dependent Akt Ser^{473} phosphorylation thus provides a surrogate assay for PI3K activity in intact cells. To explore the effects of SHP-1 on PI3K-induced Akt phosphorylation, hemagglutinin-tagged Akt (HAAkt) and Lck Y505F were co-transfected in COS7 cells and the phosphorylation of Akt examined by immunoblotting analysis using an anti-Akt antibody specifically recognizing phosphoserine 473. Results of this analysis (Fig. 4*B*) revealed Lck Y505F co-transfection to be associated with a modest increase in Akt Ser^{473} phosphorylation. By contrast, co-expression of wild-type SHP-1 with Lck Y505F and HAAkt reduced phospho-Akt to a level similar to that detected in cells transfected with HAAkt alone. Interestingly, expression of SHP-1 C453S in conjunction with Lck Y505F and HAAkt was associated with increases in levels of phospho-Akt exceeding those detected in cells expressing Lck Y505F and HAAkt (Fig. 4, *B* and *C*). These latter findings parallel the observations revealing Lck Y505F effects on p85 phosphorylation (Fig. 3*A*) to be somewhat enhanced in the context of SHP-1 C453S expression, a finding which suggests that substrate trapping by the latter protein may impact on PI3K signaling.

DISCUSSION

In the current study, the possibility that interaction between PI3K and SHP-1 contributes to the effects of these respective proteins on TCR signaling was investigated. The data reveal that SHP-1 interacts with the p85 subunit of PI3K in Jurkat T cells, and indicate this association to be enhanced by TCR stimulation. Furthermore, SHP-1 and PI3K are present in a complex including the TCR. Association of SHP-1 with p85 was also found to be inducible in COS7 cells by addition of activated Lck and to represent a phosphotyrosine-dependent interaction involving association of the p85 carboxyl-terminal SH2 domain likely with phosphorylated tyrosine 564 in the SHP-1 carboxyl-terminal tail. By further analysis of this interaction in COS7 cells, p85 was identified as a substrate for SHP-1, and the activity of tyrosine-phosphorylated PI3K shown to be markedly reduced in the presence of wild-type, but not catalytically inert SHP-1 (41). SHP-1 expression did not, however, alter lipid kinase activity of total cellular PI3K. A role for SHP-1 in regulating PI3K signaling was also evidenced by the finding that SHP-1 expression in COS7 cells engenders a decrease in phosphorylation of Akt Ser^{473}. Phosphorylation of Akt at this site involves association of the Akt PH domain with phosphorylated PI3K lipid substrates in the cell membrane and is known to be completely dependent on PI3K activation (22). Taken together, these observations provide evidence that SHP-1 not only interacts with PI3K, but also impacts upon PI3K activation and downstream signaling.

The current data indicate the SHP-1/PI3K interaction to be mediated by binding of the PI3K p85 subunit carboxyl-terminal SH2 domain to phosphorylated SHP-1 and to require that the most carboxyl-terminal located 35-amino acid segment of SHP-1 be intact. As Tyr^{564}, which has been identified as the primary target for Lck effects on SHP-1, maps within this region (18), it appears likely that Tyr^{564} represents the site on SHP-1 which interacts with the p85 SH2 domain. Interestingly, the results of these studies also revealed the truncated SHP-1 Δ35 protein to exhibit decreased phosphatase activity (data not shown), a result which contrasts with previous data

suggesting catalytic activity of this mutant form of SHP-1 to be enhanced (42). This discrepancy may reflect the differences in the conditions used for the respective phosphatase assays, the previous study involving analysis of PTP activity at pH 5.5. In the current study, the assay was performed at pH 7.3, which would presumably more closely approximate physiologic conditions. In any case, in view of the potential for this truncation mutation to alter SHP-1 activity, the SHP-1 Δ35 protein was used here only in binding studies, and its effects on p85 phosphorylation and PI3K activity were not examined.

Although p85 SH2 domains have been previously shown to specifically target YMXM phosphotyrosine motifs, the current data suggest that the carboxyl-terminal SH2 domain of p85 binds a SHP-1 phosphotyrosine residue (Tyr^{564}) embedded within a YENV motif. This divergence in the SH2 domain specificity is, however, not without precedent (30, 43). The SHP-1 SH2 domains, for example, have been demonstrated to interact with several distinct phosphotyrosine motifs (44). Furthermore, *in vitro* phosphorylation of the p85 carboxyl-terminal SH2 domain has been shown to alter its capacity to bind certain targets in activated Jurkat cells (30), a finding which again raises the possibility that the SH2 domain may interact with phosphotyrosines in more than one structural context.

Interestingly, p85 association with SHP-1 in PDGFR-stimulated MCF-7 cells has been shown to be mediated by binding of the SHP-1 amino-terminal SH2 domain to phosphorylated p85 (10). By contrast, interaction of the SHP-1 SH2 domains with phosphorylated p85 was not detected in the current study, a discrepancy which may reflect differences in the PI3K sites targeted by Lck and PDGFR, respectively (18, 45). It is also not clear whether p85 is a direct PDGFR target *in vivo*. However, taken together, these findings raise the possibility that association of SHP-1 with PI3K and the consequent modulation of PI3K signaling occurs in a variety of cell stimulatory contexts.

The data reported here concur with other data in the literature revealing the phosphorylation of p85 and the *in vitro* lipid kinase activity of immunoprecipitated PI3K to be poorly correlated (30). However, wild-type SHP-1 decreases PI3K activity in anti-phosphotyrosine immunoprecipitates and PI3K-dependent phosphorylation of Akt in intact cells. Interestingly, both p85 phosphorylation and PI3K activity, as revealed by Akt S473 phosphorylation, were found to be up-regulated in the presence of catalytically inactive SHP-1 C453S protein. As SHP-1 C453S does not enhance activity of Lck Y505F (Fig. 3*C*), these data suggest that SHP-1 C453S acts in this context as a "substrate trap," binding phosphorylated targets, but failing to dephosphorylate or release these phosphoproteins, thus protecting them from dephosphorylation by other cellular phosphatases. The increased level of phospho-Akt in the SHP-1 C453S-transfected cells may also reflect the capacity of mutant SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of PI3K bound SHP-1 C453S to induce conformational changes in PI3K which favor its activation, possibly by mimicking the effects of a positive modulator of PI3K. Both of these latter hypotheses suggest the involvement of a third molecule in the PI3K/SHP-1 interaction, a possibility also suggested by our finding that SHP-1 and PI3K can be co-immunoprecipitated from the membrane fraction of resting, serum-starved Jurkat cells in which protein phosphorylation would be expected to be minimal. Therefore, SHP-1 may also associate with PI3K by a phosphotyrosine-independent mechanism, such as interactions with an SH3 domain containing protein (46). This possibility however, remains purely speculative at present.

In summary, the data shown here reveal a functional relationship between Lck, SHP-1, and PI3K signaling proteins, which have each been identified as key elements in the induction of T cell activation. While Lck acts primarily to promote TCR signaling (6), SHP-1 effects on TCR signal relay are largely inhibitory (16, 17). The current data suggest that this inhibitory effect of SHP-1 is realized at least in part through the down-regulation of PI3K activity. However, in view of the limited understanding of the role for PI3K activity in TCR signaling, further studies are required to address the physiological significance of SHP-1 effects on PI3K. It also remains to be determined whether SHP-1 effects on PI3K signaling *in vivo* reflect direct modulation of PI3K activity by SHP-1 and/or the capacity of SHP-1 to influence other PI3K modulatory signaling effectors by virtue of its interaction with PI3K. Investigation of these various possibilities represents a promising avenue to further elucidating the mechanisms whereby both SHP-1 and PI3K impact upon the signaling cascades linking TCR stimulation to cell response.

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Tyrosine Phosphorylation of p85 Relieves Its Inhibitory Activity on Phosphatidylinositol 3-Kinase*

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Under resting conditions, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) serves to both stabilize and inactivate the p110 catalytic subunit. The inhibitory activity of p85 is relieved by occupancy of the NH_2-terminal SH2 domain of p85 by phosphorylated tyrosine. Src family kinases phosphorylate tyrosine 688 in p85, a process that we have shown to be reversed by the activity of the p85-associated SH2 domain-containing phosphatase SHP1. We demonstrate that phosphorylation of the downstream PI3K target Akt is increased in cells lacking SHP1, implicating phosphorylation of p85 in the regulation of PI3K activity. Furthermore, the *in vitro* specific activity of PI3K associated with tyrosine-phosphorylated p85 is higher than that associated with nonphosphorylated p85. Expression of wild-type p85 inhibits PI3K enzyme activity as indicated by PI3K-dependent Akt phosphorylation. The inhibitory activity of p85 is accentuated by mutation of tyrosine 688 to alanine and reversed by mutation of tyrosine 688 to aspartic acid, changes that block and mimic tyrosine phosphorylation, respectively Strikingly, mutation of tyrosine 688 to aspartic acid completely reverses the inhibitory activity of p85 on cell viability and activation of the downstream targets Akt and NFκB, indicative of the physiological relevance of p85 phosphorylation. Tyrosine phosphorylation of Tyr^{688} or mutation of tyrosine 688 to aspartic acid is sufficient to allow binding to the NH_2-terminal SH2 domain of p85. Thus an intramolecular interaction between phosphorylated Tyr^{688} and the NH_2-terminal SH2 domain of p85 can relieve the inhibitory activity of p85 on p110. Taken together, the data indicate that phosphorylation of Tyr^{688} in p85 leads to a novel mechanism of PI3K regulation.

The PI3K[1] signaling cascade has been linked to proliferation, cell survival, differentiation, apoptosis, cytoskeletal rearrangement, and vacuolar trafficking. Growth factor-responsive Class IA PI3Ks consist of heterodimers of a 110-kDa catalytic subunit associated with an 85-kDa noncatalytic regulatory subunit designated p85. The p85 adapter subunits are encoded by at least three different genes with splice variation generating multiple proteins potentially serving many different functions (1). Of the known p85 adapter subunits and splice variants, nearly all contain two Src-homology 2 (SH2) domains, which enable p85 to bind phosphotyrosine in an appropriate amino acid context. The p85 SH2 domains most frequently, but not exclusively, recognize phosphotyrosine embedded in a *YXXM* motif (2). Most p85 gene products also include a Src homology 3 (SH3) domain, as well as other domains involved in protein-protein interactions (3). All p85 family members contain a p110-binding motif located between the two SH2 domains. The diversity of protein interaction domains found among p85 family members likely contributes to the ability of multiple signaling proteins and pathways to activate PI3K. Under resting conditions, p85 serves to both stabilize p110 protein and inhibit PI3K lipid kinase activity, thereby increasing the amount of inert p110 available for activation (4). This inhibitory effect is alleviated by binding of the SH2 domains of p85, and in particular the NH_2-terminal SH2 domain, to tyrosine-phosphorylated peptides, as well as tyrosine-phosphorylated receptors or linker molecules containing the *YXXM* motif (4). Tyrosine phosphorylation of p85 binding sites within growth factor receptor cytoplasmic domains and linker molecules thus results in the recruitment of p85 to the cell membrane with consequent release of p85-mediated inhibition of PI3K (4) and colocalization of PI3K with its substrate membrane phosphatidylinositols (5) and other regulatory molecules (6, 7).

The regulatory p85 subunit of PI3K is phosphorylated at tyrosine 688 (Tyr^{688}) by the Src family kinases Lck and Abl (8) and dephosphorylated at this site by the protein tyrosine phosphatase, SHP-1 (9). While p85 is known to be tyrosine-phosphorylated in response to a variety of stimuli, the role of p85 tyrosine phosphorylation in PI3K activation is unknown (6, 10, 11). Tyrosine phosphorylation of p85 does, however, correlate with proliferative rate in Jurkat cells (12) and alters SH2 domain binding properties (8). Previous data from our group have revealed that coexpression of a constitutively active form of Lck with PI3K in COS cells results in an increase in PI3K activity (9). In this system, coincident expression of SHP-1 is associated with a decrease in PI3K activity, while expression of a phosphatase-inactive form of SHP-1 increases PI3K activity. These data suggest that phosphorylation/dephosphorylation of Tyr^{688}, a residue that maps within the p85 carboxyl SH2 domain, provides a mechanism for regulating PI3K activity. The data described herein directly address this latter possibility and demonstrate that tyrosine phosphorylation of p85 and, more specifically of Tyr^{688}, regulates PI3K activity,

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; SH, Src-homology; IL, interleukin; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGF, epidermal growth factor; GST, glutathione *S*-transferase.

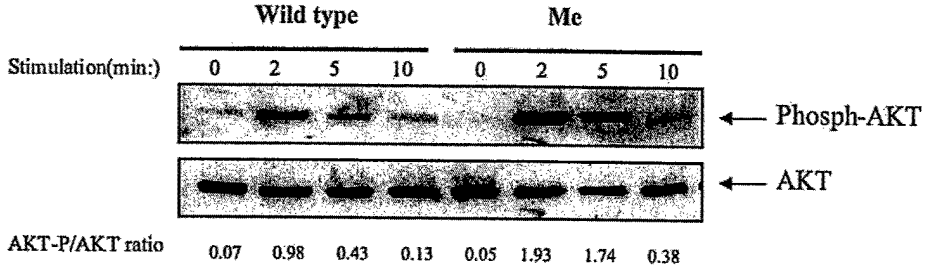

FIG. 1. **SHP-1 regulates AKT phosphorylation.** Western blot analysis showing phospho-AKT levels in total cell lysates from thymocytes from wild-type and motheaten mice (*Me*) stimulated with anti-CD3(5 μg/ml) and anti-CD28 (5 μg/ml) antibodies, followed by cross-linking with anti-hamster IgG (10 μg/ml) for the different time points indicated (*top panel*). The blot was stripped and re-probed with anti-Akt antibody as a loading control (*bottom panel*). *Numbers* below indicate the ratio of phospho-Akt/Akt band intensities as quantitated using ImageQuant software (Molecular Dynamics) and represent the results of three independent experiments.

NFκB activation, and growth factor deprivation-induced cell death. The data also link these effects of Tyr^{688} phosphorylation to the formation of an intramolecular complex with the p85 NH_2-terminal domain relieving the inhibitory effect of p85 on p110.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The anti-phosphotyrosine monoclonal antibody (4G10, IgG2B) and the rabbit polyclonal antibody against the p85 subunit of PI3K were purchased from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against Akt and phospho-Akt were purchased from New England Biolabs (Beverly, MA). A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas M. D. Anderson Cancer Center, Houston, TX). Horseradish peroxidase-goat anti-mouse IgG was purchased from Bio-Rad. The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Villette (Montreal, Quebec, Canada). The cDNA plasmids for HAAkt, and HAp85 were generous gifts of Dr. Rakesh Kumar (University of Texas M. D. Anderson Cancer Center, Houston, TX). The cDNA plasmid for HACSH2 was a generous gift of Dr. Tomas Mustelin (Laboratory of Signal Transduction, La Jolla Cancer Research Center, The Burnham Institute, La Jolla, CA), and the cDNA plasmid for pGL3/NFκB was a generous gift of Dr. David Spencer (Baylor College of Medicine, Houston, TX).

Cell Lines—COS7 cells were purchased from American Type Culture Collection (Manassas, VA). Baf/3 was a kind gift of Dr. Tada Taniguchi (University of Tokyo, Tokyo, Japan).

Cell Culture, Stimulation, and Lysis—Baf/3, MDA MB 468, and COS7 cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing penicillin/streptomycin (1%, Life Technologies, Inc.), L-glutamine (2 mM, Life Technologies, Inc.), and 10% (v/v) fetal calf serum (Sigma) at 37 °C in a humidified atmosphere. IL-3-producing cells were purchased from American Type Culture Collection (Manassas, VA). Human epidermal growth factor was purchased from Sigma. After stimulation, the cells were pelleted, resuspended in 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.4, 1 mM sodium orthovanadate, 50 mM $ZnCl_2$, 50 mM NaF, 50 mM sodium orthophosphate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and incubated at 4 °C for 20 min. After centrifugation at 14,000 × *g* for 5 min at 4 °C, postnuclear detergent cell lysates were collected.

Mutagenesis—Plasmid cDNA was mutated using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer's guidelines. All mutations were confirmed by sequencing.

Transient Transfection—Adherent cells were transfected by lipofection. Briefly, 4×10^6 cells were seeded on 100-mm cell culture plates and incubated in complete medium overnight. cDNA expression constructs were incubated in serum-free medium with LipofectAMINE (Life Technologies, Inc.) at room temperature for 30 min, then diluted with serum-free medium and incubated with cells at 37 °C for 2 h, after which time the LipofectAMINE mixture was replaced with complete medium, and the cells were returned to 37 °C for 24 h. Complete medium was then removed, the cells rinsed, and incubation continued with serum-free medium for an additional 24 h. Baf/3 cells were transfected by electroporation at 250 V and 950 microfarads.

Immunoprecipitation and Immunoblotting—Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4 °C for 2 h followed by another 2-h incubation with protein A-Sepharose beads. The immunoprecipitates were washed with immunoprecipitation wash buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Proteins were eluted from the beads by boiling in 2× Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), or anti-phosphotyrosine (1:3000), at room temperature for 2 h. Horseradish peroxidase-protein A or horseradish peroxidase-goat anti-mouse IgG was used as a secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL). Where indicated, blots were stripped by treatment with 2% SDS and 100 mM β-mercaptoethanol in Tris-buffered saline and then reprobed with desired antibodies and detected by ECL.

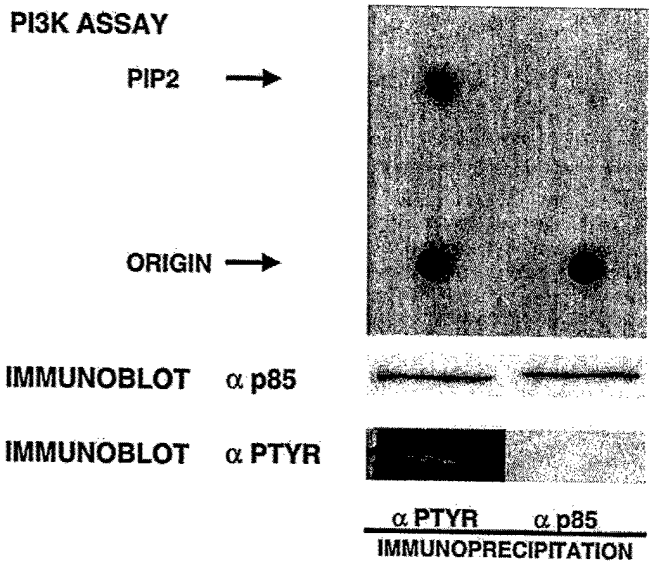

FIG. 2. **Tyrosine phosphorylation of p85 increases its specific activity.** COS7 cells were transiently transfected with a constitutively active Lck mutant Y505F. Lysates were sequentially immunoprecipitated with anti-phosphotyrosine antibodies, then with anti-p85 antibodies, and equal amounts of phosphorylated and nonphosphorylated p85 protein was subjected to a PI3K activity assay as described under "Experimental Procedures." The data are a representative example of three experiments.

Kinase Activity—Cells were lysed in 1% Nonidet P-40 lysis buffer. Cell lysates normalized for protein levels were immunoprecipitated using anti-HA or anti-p85 and protein A-Sepharose. Nontransfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described previously (9).

Assay of Akt Phosphorylation in Murine Thymocytes—To evaluate Akt phosphorylation, single cell suspensions of thymocytes were prepared from 2–3-week-old C3HeBFeJ motheaten (*me*) and wild-type control littermates derived from C3HeBFeJ *me*/+ breeding pairs maintained at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital (Toronto, Ontario, Canada). For analysis of Akt phosphorylation, 5×10^7 thymocytes were resuspended in 100 μl of RPMI 1640 medium and incubated for 30 min at 4 °C with biotinylated anti-T cell antigen receptor antibody (1 μg/ml) or anti-CD3 (5 μg/ml) plus anti-CD28 (5 μg/ml) antibody. After removal of unbound antibody, cells were resus-

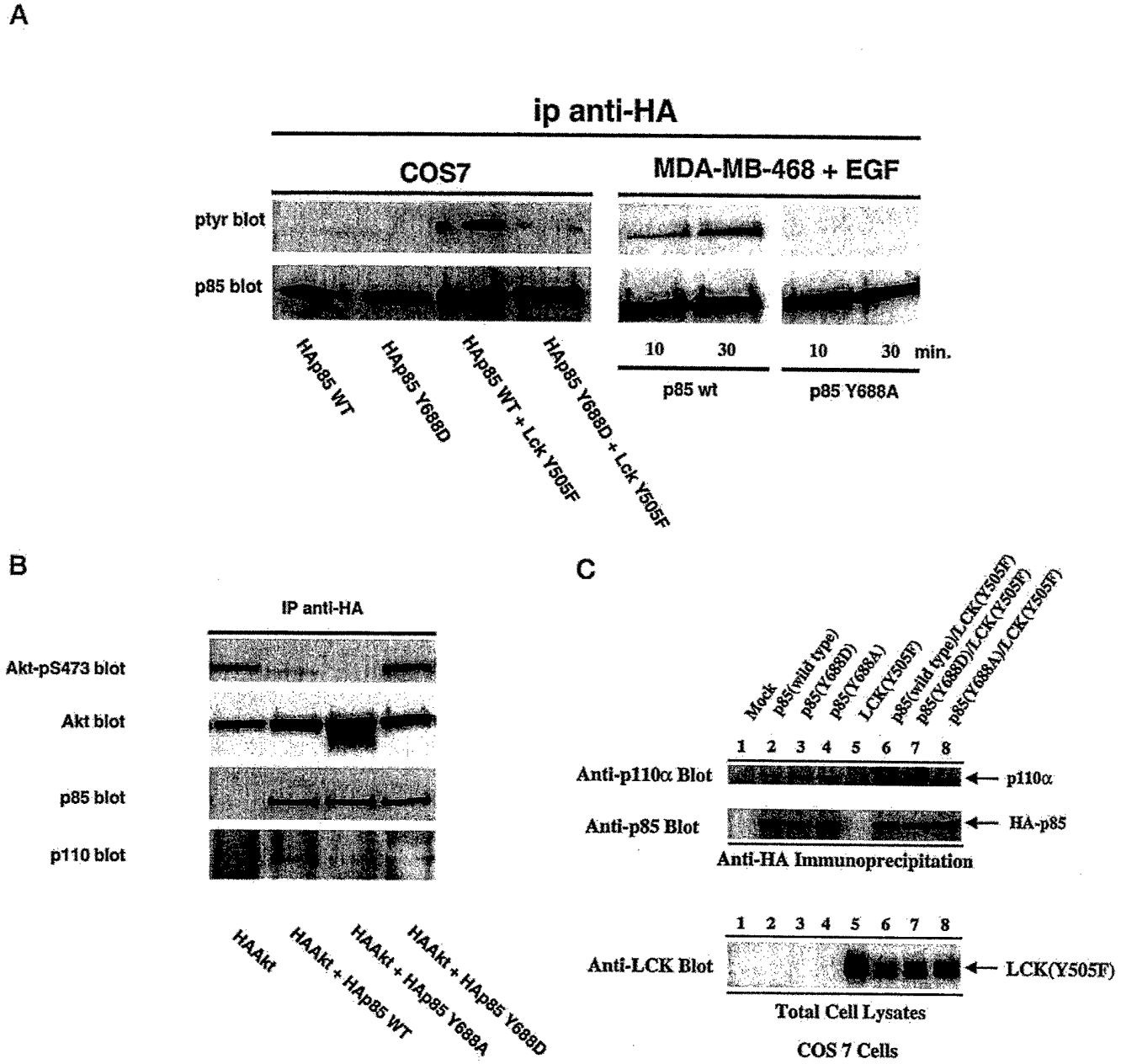

FIG. 3. **Tyrosine phosphorylation of Tyr^{688} relieves the inhibitory activity of p85 on p110.** *A*, mutation of Tyr^{688} to Asp or Ala prevents Lck505 or EGF-mediated tyrosine phosphorylation. In the *left panel*, COS7 cells were transfected with influenza virus HA epitope-tagged wild-type or Y688D HAp85 with or without Lck Y505F. Anti-HA immunoprecipitates were separated by 10% SDS-PAGE and subjected to immunoblot with anti-phosphotyrosine. In the *right panel*, p85 wild-type or Y688A were transfected into MDA-MB-468 cells, which overexpress the EGF receptor and are highly responsive to EGF. Cells were starved overnight and then incubated with EGF (50 ng/ml) for 10 or 30 min. Cells were lysed and p85 immunoprecipitated with anti-HA antibodies, resolved by SDS-PAGE, and subjected to immunoblotting with anti-phosphotyrosine antibodies. No p85 phosphorylation was detected in resting cells (not presented). *B*, p85 wild-type or Y688A, but not p85 Y688D inhibits PI3K-dependent Akt phosphorylation. HA epitope-tagged p85 wild-type, Y688A, or Y688D were coexpressed with HA epitope-tagged Akt. Lysates were immunoprecipitated with anti-HA antibodies, separated by 8% SDS-PAGE, and subjected to immunoblot with antibodies against phospho-Ser^{473} Akt, total Akt, p85, and p110. Both Akt and p85 were HA-tagged. p110 was coprecipitated with HA-p85. The data are a representative example of three independent experiments. *C*, mutation of Tyr^{688} to Asp or Ala does not affect PI3K p85 binding to p110. HA epitope-tagged wild-type, Y688D, or Y688A p85 were cotransfected with or without Lck (Y505F) in COS7 cells. Cells were serum-starved overnight prior to cell lysis. Cell lysates were subjected to anti-HA immunoprecipitation, resolved by 8% SDS-PAGE, and immunoblotted with anti-PI3K p110 antibody. The membrane was stripped and re-probed with anti-p85 antibody to confirm the expression level of HA-p85 (*upper panel*). Total cell lysates were separated by 8% SDS-PAGE and immunoblotted with anti-Lck antibody to verify the expression of Lck (Y505F) (*lower panel*). *wt* (and *WT*), wild-type.

pended at a concentration of 2×10^8 cell/ml and incubated at 37 °C for 2 min with streptavidin (10 μg/ml) or anti-hamster IgG (10 μg/ml). After antibody stimulation, cells were pelleted by 0.5-min centrifugation and then lysed and analyzed as described above.

Baf/3 Survival—Baf/3 cells were washed and reconstituted in complete medium at 11.25×10^6 cells/ml, mixed with the desired DNA, and electroporated. Samples were cultured in IL-3-free conditions for 48 h, then IL-3 was added to the sample, and the culture was continued for an additional 48 h. Triplicate 100-μl samples of each culture were transferred to a 96-well plate, mixed with 25 μl of MTT (5 mg/ml), and incubated at 37°C for 2 h. The samples were lysed with 100 μl of MTT lysis buffer (20% SDS in 50% *N,N*-dimethylformamide, pH 4.7) and absorbance at 570 nm determined.

Luciferase Assay—The luciferase assay kit was purchased from Promega (Madison, WI), and assays were carried out according to manufacturer's recommendations.

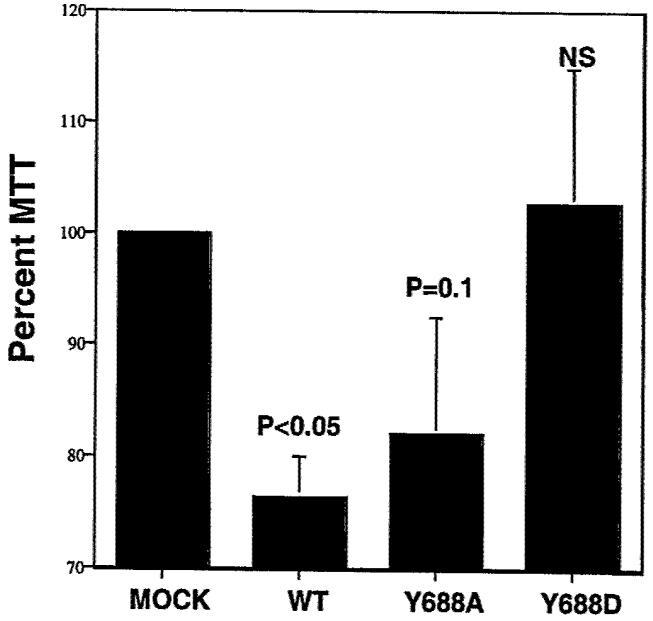

FIG. 4. **p85 wild-type or Y688A decrease survival of Baf/3 cells during growth factor deprivation.** Baf/3 cells were transiently transfected with p85 wild-type (*WT*), Y688A, or Y688D and cultured in IL-3-free conditions for 48 h. IL-3 was then added to the culture and the surviving cells expanded for an additional 48 h before MTT assay to allow surviving cells to proliferate and dying cells to clear the system. The data represent the mean ± S.E. of three experiments. *NS*, not significant.

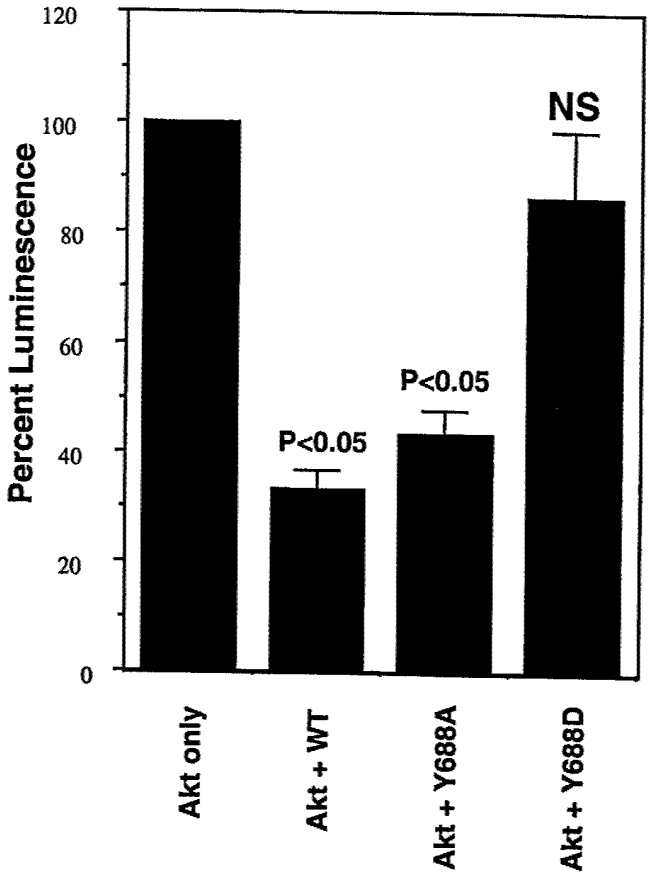

FIG. 5. **Wild-type or Y688A p85 inhibit NFκB transcriptional activity.** COS7 cells were transfected with p85 wild-type (*WT*), Y688A, or Y688D and an luciferase reporter construct that contained an NFκB consensus binding sequence. Cells were allowed to express the constructs for 48 h and luciferase activity assessed as described under "Experimental Procedures." The data are representative of mean ± S.E. of one of three experiments. *NS*, not significant.

RESULTS

Signaling through the PI3K Pathway Is Increased in Cells Lacking SHP-1—We have previously demonstrated that SHP1 associates with PI3K and dephosphorylates the kinase at tyrosine 688 (9), a residue that maps within the p85 subunit and that has been shown to be phosphorylated by Src family kinases (8). These data indicate that SHP-1 modulates p85 tyrosine phosphorylation and raise the possibility that signal transduction through the PI3K pathway can be modulated by the SHP-1 tyrosine phosphatase. To investigate this possibility, resting and T cell antigen receptor-stimulated thymocytes from SHP-1-deficient motheaten mice were evaluated for PI3K activation using an assay of Akt Ser^{473} phosphorylation as a surrogate indicator of PI3K activity. As indicated in Fig. 1, results of immunoblotting analysis revealed the level of Akt Ser^{473} phosphorylation induced in T cell antigen receptor-stimulated thymocytes to be markedly higher in motheaten compared with wild-type cells. These data indicate a role for SHP-1 in regulating not only p85 phosphorylation but also the activation of PI3K. By extension, these findings imply that the tyrosine phosphorylation status of p85 is relevant to the regulation of PI3K activity.

Phosphorylated p85 Is Associated with Higher Lipid Kinase Activity than Nonphosphorylated p85—To determine whether tyrosine phosphorylation of p85 alters the specific activity of PI3K, p85 activity was evaluated in either COS7 cells or COS7 cells transiently transfected with Lck Y505F, a constitutively active form of Lck that phosphorylates p85 at Tyr^{688} (8). To evaluate PI3K activity in relation to phosphorylation status, anti-p85 immunoprecipitates were prepared from either cell lysates subjected to preclearing with anti-phosphotyrosine antibody (*i.e.* lysates immunodepleted of tyrosine-phosphorylated species) or alternatively from anti-phosphotyrosine immunoprecipitates (so as to isolate tyrosine phosphorylated p85 species). The amount of p85 present in each sample was determined by Western blotting, and equal amounts of p85 were then assessed for lipid kinase activity using phosphatidylinositol as a substrate. As indicated in Fig. 2, this analysis revealed the enzymatic activity of p110 associated with tyrosine phosphorylated p85 (*i.e.* the p85 present in anti-Tyr(P) immunoprecipitates) to be much greater than that associated with p85 immunoprecipitated from cell lysates precleared with anti-phosphotyrosine antibody. These results suggest that PI3K lipid kinase activity is increased in association with p85 tyrosine phosphorylation and therefore provide additional evidence that PI3K activity is regulated by tyrosine phosphorylation.

p85 Y688D Expression Relieves the Inhibitory Effect of Wild-type and p85Y688A on PI3K-dependent Phosphorylation of Akt—The tyrosine residue at position 688 has previously been identified as the primary site of Lck-induced p85 phosphorylation (8). To evaluate the impact of phosphorylation at this site on PI3K activity, p85 expression constructs were derived in which Tyr^{688} was replaced by either an aspartate or an alanine residue. Due to the charged nature of aspartate, p85 Y688D protein would be predicted to mimic phosphorylated p85 protein; by contrast, Y688A cannot be phosphorylated and should therefore behave like nonphosphorylated p85 (13, 14). As indicated in Fig. 3*A*, expression of these proteins in COS7 cells or MDA MB 468 cells revealed that the p85 Y688D and the Y688A mutant proteins were not tyrosine-phosphorylated either as a consequence of Lck coexpression or activation of cells with epidermal growth factor (EGF). These data thus confirm that Tyr^{688} is the primary site of tyrosine phosphorylation in p85. To further address the relevance of Tyr^{688} phosphorylation to PI3K activation, the effects of these mutant proteins on Akt

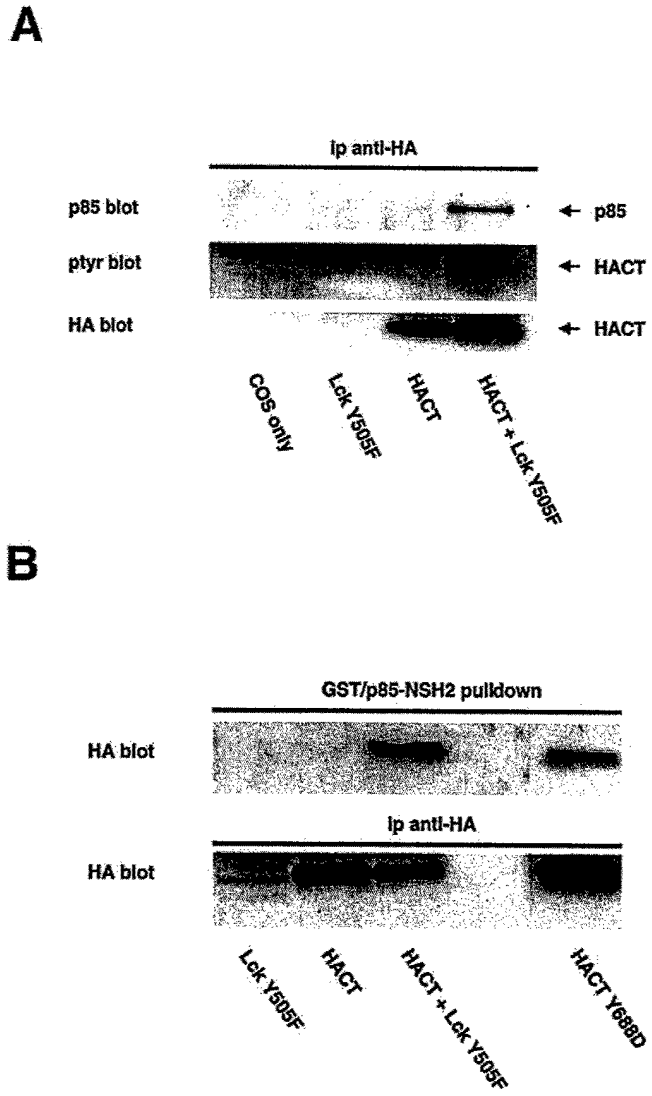

FIG. 6. **Tyrosine phosphorylated Tyr^{688} associates with the amino-terminal SH2 of p85.** *A*, tyrosine-phosphorylated carboxyl-terminal fragments of p85 associate with wild-type p85. Lysates of cells transfected HA epitope-tagged carboxyl-terminal p85 fragment (HACT), wild-type with or without Lck Y505F, were immunoprecipitated with anti-HA antibodies, separated by 10% SDS-PAGE, and subjected to immunoblot with anti-phosphotyrosine. The membrane was stripped, and reblotted with anti-p85. The membrane was stripped and reblotted with anti-HA. *B*, tyrosine-phosphorylated carboxyl-terminal fragments of p85 associate with the NH_2-terminal SH2 domain of p85 COS7 cells were transfected with wild-type HACT with or without Lck Y505F, or with HACT Y688D. The transfected cell lysates were mixed with p85 amino-terminal SH2-GST fusion protein bound to glutathione beads (*upper panel*). An equivalent amount of lysate total protein was immunoprecipitated with anti-HA and separated by SDS-PAGE along with the glutathione bead complexes and subjected to immunoblot with anti-HA (*lower panel*), demonstrating the efficacy of the interaction. The data are representative of three independent experiments.

Ser^{473} phosphorylation was also assessed. As illustrated in Fig. 3*B*, overexpression of wild-type p85 was associated with a decrease in Akt phosphorylation, a result consistent with the putative inhibitory effects of native (*i.e.* nonphosphorylated p85) on p110 kinase activity (4). This effect was further accentuated in cells expressing the tyrosine nonphosphorylatable Y688A mutant protein (Fig. 3*B*). By contrast, expression of the Y688D protein did not alter Akt phosphorylation, a result which implies that this protein facilitates p110 activation most likely by releasing the enzyme from p85-mediated inhibition. Conversion of Tyr^{688} to Ala or Asp did not alter association of p85 with p110 in the presence or absence of Lck (Fig. 3, *B* and *C*). Furthermore, Y688A and Y688D both associated equally with Cbl in the presence or absence of Lck (not presented), indicating that association with Cbl does not explain the differential effects of Y688A and Y688D on Akt phosphorylation.

Y688D Mutation Reverses the Inhibitory Effect of Wild-type p85 on Survival of IL-3-deprived Baf/3 Cells—The effects of PI3K on cell survival are mediated at least in part by activation of Akt and the consequent phosphorylation and inactivation of pro-apoptotic proteins such as BAD (15), GSK3 (16), forkhead (17), and Caspase 9 (18). To assess the relevance of p85 Tyr^{688} phosphorylation to these cellular events, the effects of wild-type and mutant p85 expression on cytokine deprivation-induced cell death were investigated using Baf/3, a cell line that undergoes apoptosis when cultured in the absence of IL-3 (19). For these studies, the IL-3-dependent Baf/3 cells were transfected with the various mutant cDNAs and then were cultured in IL-3-free medium for 48 h to induce cytokine deprivation-induced apoptosis and for an additional 48 h with exogenous IL-3 to allow surviving cells to proliferate. This provides a more sensitive assay than assessing cell number following growth factor deprivation. As shown in Fig. 4, expression of wild-type and Y688A p85 in these cells was associated with their decreased survival as compared with cells expressing vector control. Although both wild-type and Y688A induced a decrease in cell survival, the wild-type protein inhibited survival more consistently than did Y688A (Fig. 3*B*). In contrast, survival of cells expressing the Y688D mutant protein was not significantly different from that of vector control cells. Thus the expression of wild-type or Y688A p85 protein appears to inhibit PI3K activity and induce a decrease in cellular proliferation/survival, while expression of the Y688D protein has a negligible effect on cell survival. These data are therefore consistent with the contention that p85 phosphorylation modulates PI3K function and also with the capacity for aspartic acid substitution at position Tyr^{688} to disrupt p85 inhibitory effects on p110 activity.

Y688D Reverses the Inhibitory Effect of p85 on NFκB-driven Reporter Expression—To further address the functional significance of p85 phosphorylation, the effects of the various p85 mutant proteins on NFκB-directed transcription events were next evaluated. This approach was based on data revealing that activated Akt phosphorylates the IKKβ complex, resulting in the phosphorylation and consequent ubiquitination and degradation of the NFκB inhibitor IκB (20). Dissociation from IκB allows NFκB to translocate to the nucleus and participate in the formation of functional transcription complexes (20). Accordingly, the ability of the p85 mutant proteins to alter transcription of an NFκB-driven luciferase reporter construct was used as another measure of their effects on PI3K activation. As illustrated in Fig. 5, an assessment of COS7 cells transfectants expressing Akt and either p85 wild-type, Y688A, or Y688D proteins revealed that both wild-type and Y688A p85 significantly inhibited transcription of the NFκB consensus promoter ($p < 0.05$). In contrast, the Y688D mutant p85 did not inhibit NFκB-driven luciferase production. These data, which imply that Tyr^{688} phosphorylation status modulates transcription through NFκB, are again consistent with the notion that p85 phosphorylation regulates p110 enzymatic activity.

Tyr^{688} Phosphorylation Induces Association with the p85 NH_2-terminal SH2 Domain—Interaction of phosphotyrosine with the NH_2-terminal SH2 domain of p85 has been shown to relieve the inhibitory activity of p85 on p110 (4). As phosphorylation of p85 Y688 appears to have this same effect on p110, it is possible that phosphorylated Tyr^{688} interacts with the NH_2-terminal p85 SH2 domain so as to generate a structural

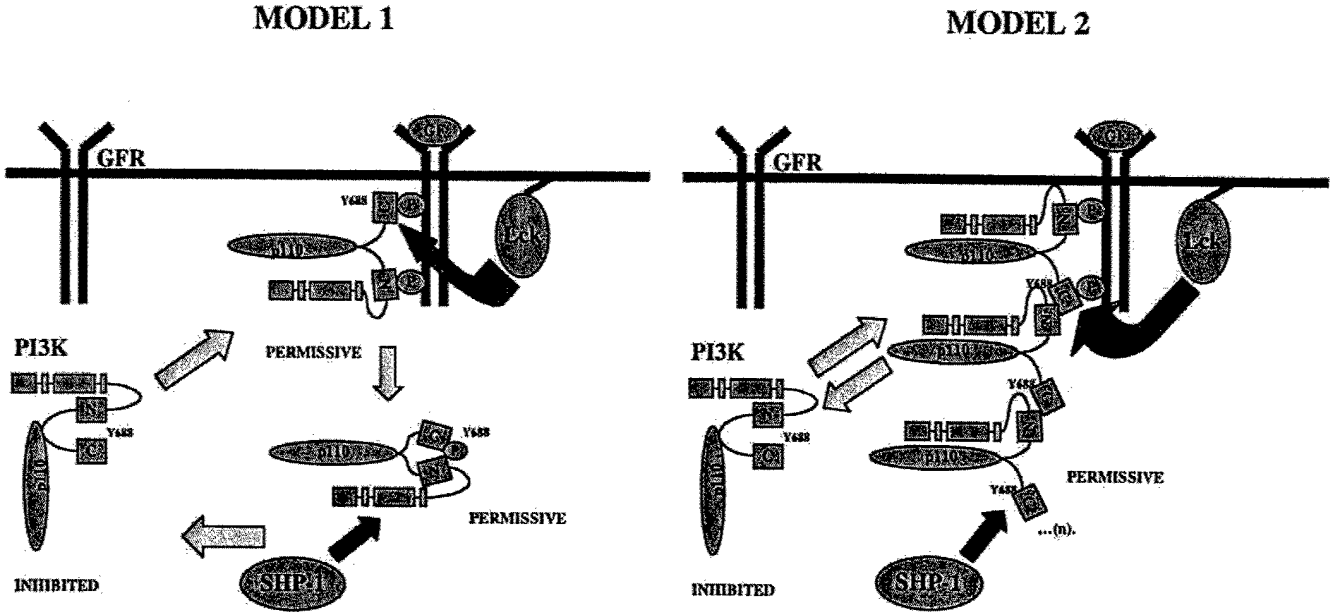

FIG. 7. **Proposed models of the effect of phosphorylation of Tyr^{688} in p85 on PI3K activity.** The *left panel* displays an intramolecular regulatory mechanism, whereas the *right panel* depicts an alternative intermolecular mechanism, resulting in PI3K concatamers. *GFR*, growth factor receptor.

arrangement that counteracts the inhibitory effects of this domain. Although Tyr^{688} does not map within the consensus binding motif for the p85 NH_2-terminal SH2 domain (YXXM), previous data have identified the capacity of nonconsensus sequences to bind to the p85 subunit (21, 22). Moreover, this type of intramolecular interaction might be facilitated by a p85 structural conformation that positions the carboxyl terminus including Tyr^{688} in close proximity to the NH_2-terminal SH2 domain of p85, a possibility predicted by previous molecular modeling data (3). Such an arrangement might evoke interaction of the NH_2-terminal SH2 domain with the tyrosine-phosphorylated Tyr^{688} residue within the COOH-terminal SH2 domain. To begin addressing this possibility, the potential for p85 tyrosine phosphorylation to promote an interaction between Tyr^{688} and the p85 NH_2-terminal SH2 domain was studied using transfected COS7 cells coexpressing Lck Y505F and an epitope-tagged p85 carboxyl-terminal fragment (HACT) encompassing the Tyr^{688} residue. The results of this analysis confirmed that Lck induces tyrosine phosphorylation of the HACT construct and revealed the capacity of endogenous p85 to coimmunoprecipitate with the tyrosine-phosphorylated p85 HACT domain but not the nonphosphorylated construct (Fig. 6*A*). The data shown in Fig. 6 also demonstrate the capacity of a glutathione *S*-transferase (GST)-linked amino-terminal SH2 domain fusion protein (NSH2-GST) to precipitate phosphorylated HACT (Fig. 6*A*) and HACT Y688D proteins but not a nonphosphorylated HACT construct. Together, these findings suggest that the amino-terminal SH2 domain of p85 associates with tyrosine-phosphorylated Tyr^{688}. As shown in Fig. 6*B*, mutation of tyrosine 688 to aspartate (to mimic tyrosine phosphorylation) allows the association between HACT and NSH2-GST protein to ensue in the absence of Lck Y505F, an observation that confirms the involvement of phosphorylated Tyr^{688} in this association and rules out the possibility that Lck Y505F acts as a linker in coupling these domains together.

DISCUSSION

The biochemical events governing protein tyrosine phosphorylation are central to the regulation of cellular signaling in all eukaryotic cells. However, while a myriad of intracellular proteins undergo tyrosine phosphorylation following cell stimulation, for many proteins, the effects of phosphorylation on function are not well defined. This latter group of proteins includes PI3K, an enzyme that is inducibly tyrosine-phosphorylated in many biological contexts. It has been suggested that PI3K is negatively regulated by serine autophosphorylation of the p85 regulatory subunit (23). However, interaction of the p85 SH2 domains with tyrosine-phosphorylated peptides appears to alleviate this inhibition, a finding that implies a role for tyrosine phosphorylation in regulating PI3K activity (4). This possibility is strongly supported by the current data showing that SHP-1, an enzyme that dephosphorylates the major tyrosine phosphorylation site on p85, Tyr^{688}, down-regulates the PI3K/Akt activation pathway. Moreover, the current data, revealing lipid kinase activity to be higher in the p85 protein present in anti-phosphotyrosine immunoprecipitates than in the p85 protein immunoprecipitated from cell lysates immunodepleted for tyrosine phosphorylated species, also indicate a direct relationship between p85 phosphorylation status and PI3K activity. Enhanced PI3K activity in this latter experiment implies that the inhibitory effect of the p85 SH2 domains on enzymatic activity has been released, a phenomenon that might relate to the tyrosine phosphorylation of p85 *per se* or, alternatively, to interactions of the p85 SH2 domains with tyrosine-phosphorylated proteins captured by anti-phosphotyrosine immunoprecipitation. To distinguish between these possibilities, p85 proteins mutated at the major tyrosine phosphorylation site (Tyr^{688}) were investigated with respect to their effects on PI3K activity. The results of this analysis revealed p85 Y688A protein, which cannot be phosphorylated at Tyr^{688}, to be associated with impaired PI3K activity as manifested by decreases in

Akt phosphorylation, BAF/3 cell survival, and NFκB promoter activation. By contrast, these latter activities were all enhanced in cells expressing a mutant p85 protein, Y688D, which is predicted to mimic tyrosine-phosphorylated p85. Taken together, these data provide compelling evidence that PI3K activity is regulated by phosphorylation of p85 at position Tyr^{688}.

While the crystal structure of full-length p85 bound to phosphopeptide is not currently available, the predicted protein sequence of the intervening iSH2 domain (p110 binding site between the two SH2 domains) indicates a pair of antiparallel helices and thus predicts that the two SH2 domains are closely aligned (3). These data raise the possibility of an intramolecular association involving binding of the phosphorylated Tyr^{688} residue within the p85 carboxyl-terminal tail to the p85 amino-terminal SH2 domain. This model, which is illustrated in Fig. 7, is supported by the current data, which reveal the ability of full-length p85 to associate with the phosphorylated, but not nonphosphorylated, Tyr^{688}-containing carboxyl-terminal fragment of p85 and which also suggest that this association is mediated via the p85 amino-terminal SH2 domain (Fig. 6). The data also exclude the possibility that this association depends upon Lck functioning as an intermediary "linker" protein, as the association occurs in the absence of Lck when p85 Y688D is used in the analysis. Together, these data suggest the existence of an intramolecular interaction, between phosphorylated Tyr^{688} and the amino-terminal SH2 domain of p85 (Fig. 7).

Although the amino acid sequence surrounding Tyr^{688} does not conform to the expected p85 SH2 target sequence (YXXM), this SH2 domain has already been shown to exhibit flexibility in terms of the target motif (21, 22). Furthermore, an intramolecular association of the nature proposed here may provide a mechanism to prevent binding of the p85 SH2 domains to low affinity substrates. This possibility is supported by previous data revealing p85 association with several phosphorylated proteins to be disrupted upon Tyr^{688} phosphorylation (8). The current data suggest that this latter observation may reflect competitive inhibition consequent to the formation of an intramolecular association. As with SH2 occupation by other phosphopeptides, this association would serve to "relax" the p85-mediated inhibition of p110 PI3K activity. In addition to this model, the current data might also be explained by another model wherein phosphorylation of Tyr^{688} triggers an intermolecular interaction between individual p85 proteins, again inducing disruption of the inhibitory activity of p85 (Fig. 7). In this alternative "PI3K concatamer" model, the recruitment of multiple PI3K molecules could represent a mechanism whereby the PI3K signaling cascade is amplified. It is possible that p85 intramolecular interactions also promote PI3K signal amplification by facilitating the removal of phosphorylated PI3K and thus freeing the receptor for subsequent association with a new PI3K. The newly detached, phosphorylated PI3K could then be dephosphorylated by SHP-1 and returned to a basal state, once again available for recruitment to a phosphorylated receptor. Alternatively, an induced intramolecular interaction may represent a mechanism by which PI3K is removed from activated growth factor receptors. Recent studies have shown that an intermolecular interaction also occurs between the p85 SH3 and proline-rich domains (24), a result which suggests that concatamers of p85 may play a role in forming multimeric interaction complexes. Whichever model proves valid, the capacity of Y688D to mimic the effect of Tyr^{688} phosphorylation implies that the minimal requirement for this association is phosphorylation at p85 Tyr^{688}.

Taken together, the data indicate that phosphorylation of 688 relieves the inhibitory activity of p85 on p110 and suggest that this effect is mediated by the association of phosphorylated tyrosine 688 with the NH_2-terminal SH2 domain of p85. Thus intramolecular interactions with phosphorylation sites in p85 have the potential to contribute to the outcome of ligand activation of cells.

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Inhibition of growth-factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in breast cancer cells

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The protein kinase B/Akt serine/threonine kinase, located downstream of phosphoinositide 3-kinase (PI-3K), is a major regulator of cellular survival and proliferation. Atypical protein kinase C (aPKC) family members are activated by PI-3K and also contribute to cell proliferation, suggesting that Akt and aPKC might interact to activate signalling through the PI-3K cascade. Here we demonstrate that blocking PKC activity in MDA-MB-468 breast cancer cells increased the phosphorylation and activity of Akt. Functional PI-3K was required for the PKC inhibitors to increase Akt phosphorylation and activation, potentially owing to the activation of specific PKC isoforms by PI-3K. The concentration dependence of the action of the PKC inhibitors implicates aPKC in the inhibition of Akt phosphorylation and activity. In support of a role for aPKC in the regulation of Akt, Akt and PKCζ or PKCλ/ι were readily co-precipitated from the BT-549 breast cancer cell line. Furthermore, the overexpression of PKCζ inhibited growth-factor-induced increases in Akt phosphorylation and activity. Thus PKCζ associates physically with Akt and decreases Akt phosphorylation and enzyme activity. The effects of PKC on Akt were transmitted through the PI-3K cascade as indicated by changes in p70 s6 kinase ($p70^{s6k}$) phosphorylation. Thus PKCζ, and potentially other PKC isoenzymes, regulate growth-factor-mediated Akt phosphorylation and activation, which is consistent with a generalized role for PKCζ in limiting growth factor signalling through the PI-3K/Akt pathway.

Key words: atypical PKC, epidermal growth factor, phosphoinositide 3-kinase, PKC inhibitor, $p70^{s6k}$.

INTRODUCTION

Three mammalian isoforms of protein kinase B (PKB)/Akt have been identified, termed PKBα, PKBβ and PKBγ, or Akt1, Akt2 and Akt3. Akt1 and Akt2 are widely expressed in various tissues, whereas the expression of Akt3 is more restricted [1]. Akt regulates multiple biological functions including protein synthesis, apoptosis and glycogenesis [2,3]. The amplification of multiple components of the phosphoinositide 3-kinase (PI-3K) signalling cascade, including the catalytic subunit of PI-3K and Akt2 and mutational inactivation of the PTEN multifunctional phosphatase in human carcinomas, is in accordance with the importance of Akt and PI-3K in tumorigenesis [1].

The activation of Akt by growth factors is mediated, at least in part, by phosphorylation of Thr-308 in the catalytic domain and Ser-473 at the C-terminus. In many systems the phosphorylation of both Akt sites is blocked by pretreatment of the cells with the PI-3K inhibitors wortmannin or LY294002, indicating that they are phosphorylated as a consequence of PI-3K signalling [4,5]. PtdIns(3,4,5)P_3-dependent protein kinase 1 (PDK1) specifically phosphorylates Thr-308, whereas PDK-2 phosphorylates Ser-473. Ilk, PDK1 or autophosphorylation could account for the phosphorylation of Ser-473 [6–8]. PI-3K, by the generation of 3-phosphorylated PtdIns(3,4,5)P_3 and subsequent conversion into PtdIns(3,4)P_2 by SHIP, initiates a kinase cascade converging on Akt. In addition, heat shock and the activation of adenylate cyclase can modulate Akt activity by PI-3K-independent mechanisms [9]. A recent report showed that protein kinase Cζ (PKCζ) might negatively regulate Akt activity in a PI-3K-independent manner [10]. Thus both PI-3K-dependent and PI-3K-independent pathways might contribute to the regulation of Akt, depending on the ligand and the cell lineage analysed.

PKC is a family of structurally related serine/threonine protein kinases. The mammalian PKC isotypes have been grouped into three subfamilies, namely classical PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs (aPKCs), on the basis of their structural and regulatory properties. PI-3K regulates aPKC, potentially through the activation of PDK1 by 3-phosphorylated phosphatidylinositols [11]. The multiple mechanisms of activation of PKC might account for the pleiomorphism and diversity of the cellular activities in which PKC has been implicated. It is also likely that specific PKC isoenzymes execute distinct cellular functions, including the regulation of anchorage-dependent and anchorage-independent growth, alterations in morphology and tumorigenicity [12–14]. The aPKC isotypes have been shown to be critically involved in cell proliferation and survival [15,16].

Here we demonstrate that PKCζ and, to a smaller extent PKCλ/ι, physically and functionally interact with Akt. This interaction is associated with an inhibition of activation-dependent phosphorylation of Akt at both Ser-473 and Thr-308. This translates into a decrease in Akt activity and a subsequent decrease in phosphorylation at Thr-389 in p70 s6 kinase ($p70^{s6k}$) in intact cells. This process is PI-3K-dependent, probably as a consequence of PI-3K-dependent activation of PKCζ. Thus the

Abbreviations used: aPKC, atypical PKC; cPKC, classical PKC; dn, dominant-negative; EGF, epidermal growth factor; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; mAb, monoclonal antibody; nPKC, novel PKC; $p70^{s6k}$, p70 s6 kinase; PDK1, PtdIns(3,4,5)P_3-dependent protein kinase 1; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C.

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activation of PKCζ through PI-3K serves to limit signalling through the PI-3K-Akt signalling cascade.

EXPERIMENTAL

Antibodies and reagents

Rabbit anti-PKCδ, anti-PKCε, anti-PKCη, anti-PKCι, anti-PKCζ and mouse anti-PKC (MC5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(phospho-Akt), anti-(phospho-extracellular-signal-regulated protein kinase) and anti-(phospho-p70^{s6k}) antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Monoclonal anti-HA was a gift from Dr Bing Su (University of Texas M. D. Anderson Cancer Center, Houston, TX, U.S.A.). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from Bio-Rad (Hercules, CA, U.S.A.). HRP-conjugated Protein A was obtained from Amersham (Arlington Height, IL, U.S.A.). Protein-A-conjugated Sepharose 4B was purchased from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Ro-31-8220, GF109203X and LY294002 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Wortmannin and monoclonal anti-(epidermal growth factor receptor) antibody were obtained from Sigma (St Louis, MO, U.S.A.). HA-epitope tagged wild-type Akt was a gift from Dr Julian Downward (London, U.K.). cDNA plasmids of PKCζ and PKCλ/ι were purchased from Invitrogen (Carlsbad, CA, U.S.A.). For the construction of a kinase-deficient dominant-negative (dn) PKCζ K275W mutant, site-directed mutagenesis of the full-length PKCζ cDNA was performed as described previously [17]. 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (lysophosphatidic acid) 18:1 (LPA 18:1) was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Cell lines

Human breast cancer cell lines MDA-MB-468 (University of Texas M. D. Anderson Cancer Center, Houston, TX, U.S.A.) and BT-549 from American Type Culture Collection (Manassas, VA, U.S.A.) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) containing 1% (w/v) penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 10% (v/v) fetal bovine serum (Sigma) at 37 °C in a humidified atmosphere.

Transient transfection

Cells were transiently transfected with various combinations of the expression vectors by Fugene™ 6 Transfection Reagent (Boehringer Mannheim Inc., Indianapolis, IN, U.S.A.) as recommended by the manufacturer.

Cell lysis, immunoprecipitation and immunoblotting

After transfection, cells were serum-starved overnight before stimulation with epidermal growth factor (EGF) (20 ng/ml) or LPA (5 μM) for 10 min. Inhibition of PKC activity was performed by treatment of the cells with different doses of Ro-31-8220 or GF109203X for 1 h before cell harvest. PI-3K activity was inhibited by pretreatment of the cells with LY294002 or wortmannin for 1 h before stimulation. Cells were washed twice with cold PBS and lysed in ice-cold lysis buffer [50 mM Hepes (pH 7.4)/150 mM NaCl/1 mM EGTA/100 mM NaF/1.5 mM $MgCl_2$/10 mM sodium pyrophosphate/1% (v/v) Triton X-100/1 mM Na_3VO_4/10% (v/v) glycerol/1 mM PMSF/10 μg/ml aprotinin]. Cellular protein concentration was determined by bicinchoninic acid reaction (Pierce, Rockford, IL, U.S.A.). For immunoprecipitation, detergent lysates were incubated with 1 μg of anti-HA mAb (monoclonal antibody) at 4 °C for 2 h. Immune complexes were captured by Protein A–Sepharose beads. Immunoprecipitates were washed with IP wash buffer [0.5% (v/v) Triton X-100/0.5% (v/v) Nonidet P40/10 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM Na_3VO_4/1 mM PMSF]. Proteins were separated by SDS/PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA, U.S.A.). Membranes were blocked with 5% (w/v) BSA for 1 h and then incubated for 2 h at room temperature or overnight at 4 °C with anti-(phospho-Akt) (1:1000 dilution), total Akt antibody (1:1000 dilution), anti-(phospho-p70^{s6k}) (1:1000 dilution) or PKC isoform antibodies (0.5 μg/ml). Membranes were washed in TBS-T buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1% (v/v) Tween 20] and incubated with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:2500 dilution) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (ECL®; Amersham).

Akt kinase activity

Cells were lysed in 1% (v/v) Nonidet P40 lysis buffer. Cell lysates, normalized for protein levels (bicinchoninic acid assay), were immunoprecipitated with anti-(total Akt) and Protein A–Sepharose. Akt kinase activity was determined as described [4].

RESULTS

Expression of PKC isoforms in breast cancer cell lines MDA-MB-468 and BT-549

To investigate a potential role of PKC in regulating the PI-3K signalling pathway in breast cancer cells, we first assessed the expression of different PKC isoforms in MDA-MB-468 and BT-549 breast cancer cell lines. Both cell lines expressed the δ, ε, η, ζ and λ/ι isoforms of PKC, with MDA-MB-468 cells having higher levels of each of these isoforms than BT-549 cells (Figure 1). Furthermore, the mAb MC-5, which recognizes all cPKC isoenzymes, failed to detect cPKC expression in MDA-MB-468 total cell lysates. Ready detection of cPKC in BT-549 cells suggests that MDA-MB-468 cells express low to absent levels of conventional PKC isoforms.

Inhibition of aPKC increases Akt phosphorylation and activity

To explore the regulatory effects of PKC on Akt, we assessed the effect of the PKC inhibitor Ro-31-8220 on Akt phosphorylation in MDA-MB-468 breast cancer cells, given that MDA-MB-468 cells expressed nPKCs and aPKCs but lacked substantive expression of cPKCs (Figure 1). Ro-31-8220 is a potent PKC inhibitor, binding to PKC isoforms competitively with ATP [18]. As shown in Figure 2, Ro-31-8220 at 1–6 μM significantly increased both Ser-473 and Thr-308 phosphorylation of Akt, reaching maximum effects at 6 μM. In contrast, the expression level of Akt was not altered by Ro-31-8220. As predicted by the increase in Ser-473 and Thr-308 phosphorylation of Akt, Ro-31-8220 enhanced Akt enzyme activity as assessed in kinase assays *in vitro* with histone 2B as substrate.

Because Ro-31-8220 demonstrates little selectivity for specific PKC isoforms [18] and might inhibit other kinases, this result raised the question of which subtype(s) of PKC could contribute to the regulation of Akt activity. As little or no cPKC is expressed in MDA-MB-468 cells, the effect of Ro-31-8220 on Akt phosphorylation and activation (Figure 2) is unlikely to be mediated by cPKCs.

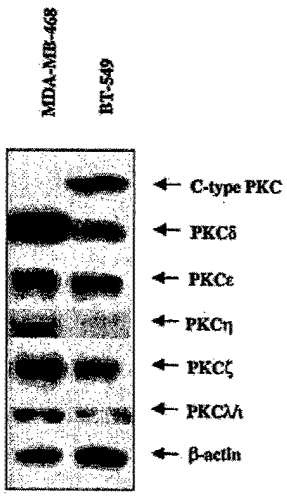

Figure 1 Expression of PKC isoforms in BT-549 and MDA-MB-468 breast cancer cell lines

Cells were cultured and harvested as described in the Experimental section. Cells were lysed; proteins were then separated by SDS/PAGE and immunoblotted with mAb MC-5 (against cPKCs) or polyclonal rabbit anti-PKCδ, anti-PKCε, anti-PKCη, anti-PKCζ or anti-PKCλ/ι antibodies. The same membranes were stripped and reprobed with anti-(β-actin) mAb to confirm equal loading of proteins. The immunoblots were detected with enhanced chemiluminescence reagents. The results shown are representative of three independent experiments.

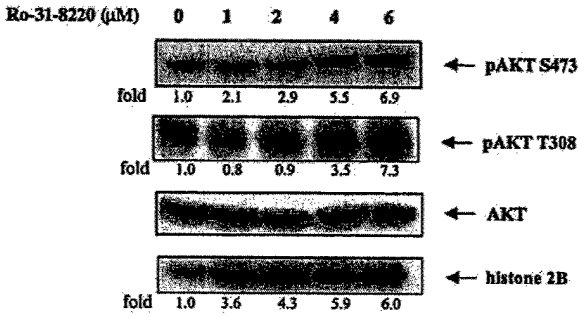

Figure 2 Increase in Akt phosphorylation and activation by Ro-31-8220 in a dose-dependent manner

MDA-MB-468 cells were starved overnight, then treated with the indicated amounts of Ro-31-8220 or vehicle [0.1% (v/v) DMSO] for 1 h. Cells were lysed and the samples were processed for Western blotting with anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies. The same membranes were stripped and re-probed with anti-Akt antibody to detect the expression levels of Akt. For assessment of Akt kinase activity, cells treated with Ro-31-8220 were lysed and Akt kinase activity was determined as described in the Experimental section. The assay mixture was separated by SDS/PAGE [12% (w/v) gel] and ^{32}P-labelled product was detected by autoradiography. Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of at least four independent experiments.

To gain further insight into the relative role of aPKCs and nPKCs in Akt regulation, we used GF109203X, which at a concentration of 0.2 μM is sufficient to block approx. 90% of the kinase activity of cPKC and nPKC isoenzymes, and for aPKCs its IC_{50} is 6–10 μM [19,20]. Incubation of MDA-MB-468 cells with GF109203X at 0.1, 1, 2, 5 and 10 μM increased Akt phosphorylation at Thr-308 by 3.2-fold, 3.7-fold, 6.7-fold, 7.3-fold and 12.6-fold respectively; similar results were seen with Ser-473 (Figure 3). These results are most compatible with aPKCs' being major negative regulators of Akt. However, the concentration dependence of GF109203X also suggests that nPKC might contribute to the regulation of Akt phosphorylation and activity.

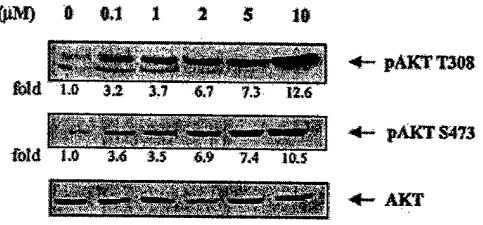

Figure 3 GF109203X increases Akt phosphorylation

For dose–response studies, serum-starved MDA-MB-468 cells were incubated for 1 h with the indicated amounts of GF109203X. Cells were then lysed; proteins were then separated by SDS/PAGE and immunoblotted with anti-(phospho-Thr-308) (top panel) or anti-(phospho-Ser-473) (middle panel) antibodies. The membranes were stripped and reprobed with anti-AKT to confirm equal expression levels of Akt (bottom panel). Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of two independent experiments.

Activation of Akt by Ro-31-8220 is PI-3K-dependent

To determine whether activation of Akt by Ro-31-8220 was dependent on PI-3K activity, we pretreated MDA-MB-468 cells with PI-3K inhibitors LY294002 (1 μM) or wortmannin (10 nM and 100 nM) for 1 h before incubation of the cells with Ro-31-8220 (4 μM, 1 h). Western blot analysis with anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies demonstrated that inhibition of PI-3K activity inhibited Ro-31-8220-induced Akt phosphorylation and decreased basal Akt phosphorylation (Figure 4). Similar results were obtained when Akt enzyme activity was assessed (Figure 4). In contrast, pretreatment with rapamycin did not affect basal and Ro-31-8220-induced Akt phosphorylation and activation (Figure 4, lower panel), ruling out the involvement of the FRAP/mTOR (mammalian target of rapamycin) phosphatidylinositol kinase-related kinase, which is also sensitive to wortmannin and LY294002 [21]. When approximately 90% of basal Akt phosphorylation was inhibited by 1 μM LY294002, as indicated in Figure 4 (upper panel), Ro-31-8220 induced a small but detectable increase in Akt phosphorylation, suggesting that a small PI-3K-independent effect of Ro-31-8220 might exist. However, the complete inhibition of Ro-31-8220-induced Akt phosphorylation and activation by 100 nM wortmannin indicates that most of the effect of Ro-31-8220 is dependent on PI-3K.

Stable association of PKCζ and Akt in breast cancer cells

PKCζ and Akt have been demonstrated to associate *in vitro* [22,23] and in intact cells [3,10] providing a potential mechanism for the effects of PKC inhibitors on Akt phosphorylation and activity. To investigate the possible functional link between aPKC isoforms and Akt in breast cancer cell lines, we examined the physical association of exogenous PKCζ or PKCλ/ι with Akt in BT-549 breast cancer cells, because BT-549 cells expressed lower levels of aPKCs than MDA-MB-468 cells (Figure 1). We co-transfected BT-549 cells with expression constructs encoding

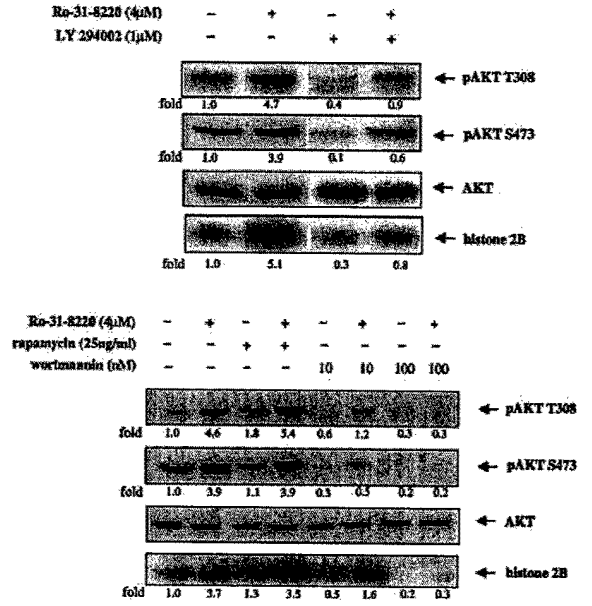

Figure 4 PI-3K dependence of Ro-31-8220-induced Akt phosphorylation and activation

Serum-starved MDA-MB-468 cells were pretreated for 1 h with LY294002 (upper panel) or rapamycin or wortmannin (lower panel), then incubated for 1 h with 4 μM Ro-31-8220. Cells were lysed; lysates were separated by SDS/PAGE [10% (w/v) gel], then immunoblotted with anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies. Equal loading of proteins was determined by assessing Akt levels with anti-Akt antibody. Equal amounts of cell lysates were subjected to Akt kinase assay as described in the Experimental section. ^{32}P-labelled products were separated by SDS/PAGE [12% (w/v) gel] and detected by autoradiography. Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of three independent experiments.

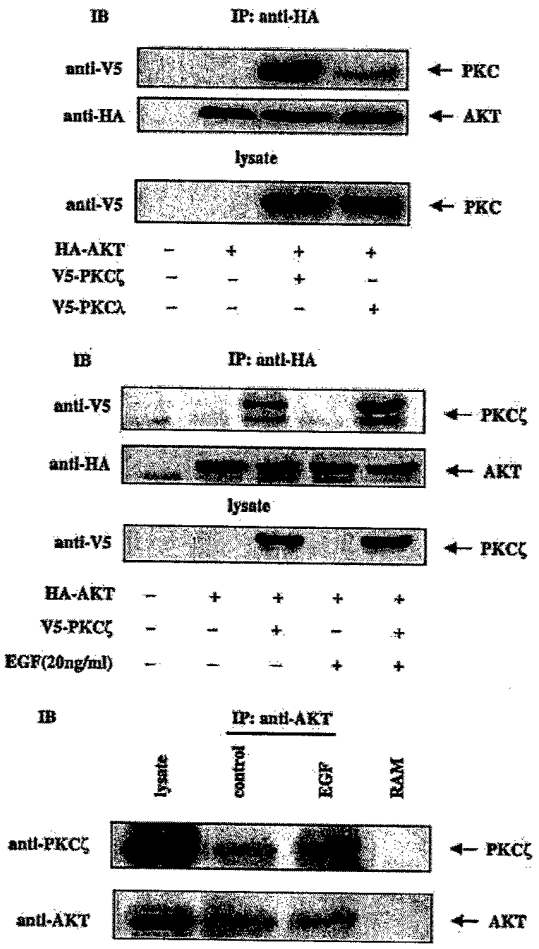

Figure 5 Co-immunoprecipitation of PKCζ with Akt

Top panel: BT-549 cells were cultured in a 60 mm dish until 80% confluent; they were then co-transfected with HA-Akt and V5-PKCζ or V5-PKCλ/ι at a ratio of 1:3 as described in the Experimental section, to ensure that all HA-Akt-containing cells also expressed PKCζ. Co-transfection of HA-Akt with empty vector (pCDNA3.1 GS) was performed as a control. At 24 h after transfection, cells were serum-starved overnight. Cells were lysed and cell lysates were subjected to immunoprecipitation (IP) with anti-HA mAb and subsequent immunoblotting (IB) with anti-V5 antibody. Equal loading of proteins was detected with anti-HA mAb. The transfection efficiency of PKCζ or PKCλ/ι was confirmed by immunoblotting whole cell lysates with anti-V5 antibody. Middle panel: BT-549 cells were co-transfected with HA-Akt and V5-PKCζ at a 1:3 ratio. After transfection, cells were serum-starved overnight followed by 10 min of stimulation with EGF (20 ng/ml) or no stimulation before lysis. HA-Akt was immunoprecipitated (IP) from cell lysates with anti-HA mAb. The presence of PKCζ in the immunoprecipitates was detected by immunoblotting (IB) with anti-V5 antibody, as indicated. The same membrane was stripped and reprobed with anti-HA mAb to confirm equal protein loading. Whole cell lysates were evaluated for expression of V5-PKCζ with anti-V5 mAb antibody. Bottom panel: endogenous Akt was immunoprecipitated (IP) with anti-Akt antibody from 500 μg of BT-549 cell lysate either untreated or treated with EGF (20 ng/ml) for 10 min. The presence of endogenous PKCζ was detected by immunoblotting (IB) with anti-PKCζ antibody. Rabbit anti-mouse (RAM) antibody was used as a negative control in the experiment. The results shown are representative of three independent experiments.

V5-tagged PKCζ or V5-tagged PKCλ/ι with a construct encoding HA-Akt and then assessed co-immunoprecipitation of the transfected PKC with Akt. As demonstrated in Figure 5 (top panel), the exogenous PKCζ was readily detected in Akt immunoprecipitates. PKCλ/ι was present in Akt immunoprecipitates but at lower levels than PKCζ despite similar levels of expression of PKCλ/ι, PKCζ and Akt in each transfection. Strikingly, EGF increased the ability to detect PKCζ in Akt immunoprecipitates approx. 5-fold, indicating that the interaction between these mediators was stabilized by activation of the EGF receptor (Figure 5, middle panel). Next we evaluated the ability of endogenous PKCζ and Akt to interact in breast cancer cells. As illustrated in Figure 5 (bottom panel), an EGF-dependent binding of endogenous PKCζ with Akt was readily observed in BT-549 cells, suggesting a direct association-mediated interaction of the two signalling proteins in intact cells. Thus the aPKCs PKCζ and, to a smaller extent, PKCλ/ι bind Akt, a process that is increased by the activation of cells by EGF. This is in contrast with a previous report that did not assess the interaction of PKCλ/ι with Akt and demonstrated a growth-factor-dependent dissociation of PKCζ and Akt in transfected cells [10].

Wild-type PKCζ, but not dn PKCζ, decreases EGF-induced Akt phosphorylation at Ser-473 and Thr-308

Phosphorylation of Akt at Ser-473 and Thr-308, which is indicative of Akt activation [4,24], was used to assess whether the physical interaction of PKCζ with Akt altered Akt activity. As shown in Figure 6 (top panel), EGF treatment induced a modest increase in Akt phosphorylation at Ser-473 and Thr-308 in cells transfected with Akt. In contrast, a marked decrease in Akt

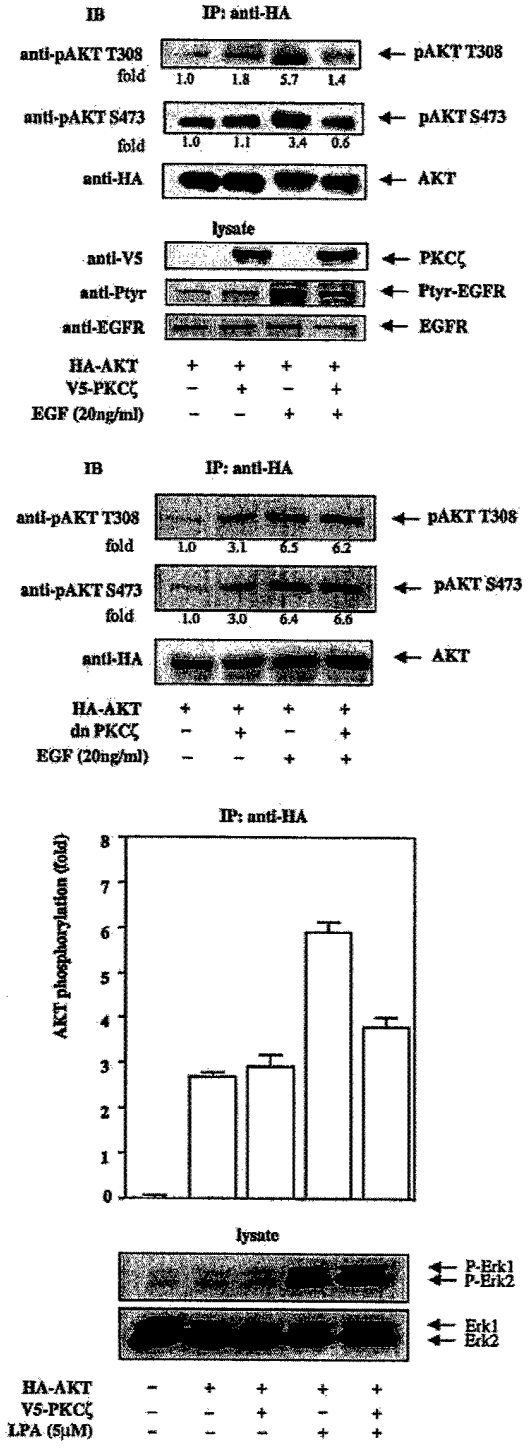

Figure 6 Akt phosphorylation induced by EGF or LPA is reversed by overexpression of wild-type PKCζ, but not dn PKCζ

The same co-transfection protocol was used as described in the legend to Figure 5 with either V5-PKCζ (top panel) or dn V5-PKCζ (middle panel). BT-549 cells were serum-starved overnight after transfection and were either left unstimulated or stimulated with EGF (20 ng/ml) for 10 min. Cells were lysed and HA-Akt was immunoprecipitated (IP) from the cell lysates by anti-HA mAb. The immunoprecipitates were separated by SDS/PAGE [8% (w/v) gel] and immunoblotted (IB) with rabbit anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies. Equal loading of proteins and equal transfection efficiency were detected as indicated previously. Phosphorylation of the EGF receptor was examined by Western blotting of whole cell lysate with anti-phosphotyrosine mAb (top panel). Results were quantified by densitometry and the value from empty-vector-transfected and untreated cells was taken as unity. Bottom panel: equal amounts of HA-Akt were immunoprecipitated (IP) from lysates from MDA-MB-468 cells co-transfected with either empty vector or V5-PKCζ with HA-Akt, then either unstimulated or stimulated with LPA (5 μM) for 10 min as indicated. Phosphorylation of HA-Akt was detected by immunoblotting the immunoprecipitates with anti-(phospho-Ser-473) polyclonal antibody; the densitometry is presented as a histogram. The phosphorylation of ERK1 and ERK2 was examined by Western blotting of cell lysate with anti-(phospho-ERK) antibody. Values are means ± S.D. for three independent experiments.

phosphorylation was observed in EGF-treated cells co-transfected with HA-Akt and wild-type PKCζ. The expression of exogenous PKCζ and Akt without altering the ability of EGF to activate the EGF receptor was determined by Western blotting of whole cell lysate with phosphotyrosine antibody as indicated in Figure 6 (top panel).

Expression of PKCζ had little effect on basal Akt phosphorylation (Figure 6, top panel), potentially arguing that PKCζ must be activated as a consequence of PI-3K for its activity to be manifest. To clarify this issue, we exploited dn PKCζ, which has the critical lysine residue at the ATP-binding site replaced by tryptophan, resulting in a kinase-defective mutant [17], to inhibit the activity of endogenous PKCζ. As illustrated in Figure 6 (middle panel), an approx. 3-fold increase in Akt phosphorylation at both Thr-308 and Ser-473 was observed in MDA-MB-468 cells co-expressing dn PKCζ and HA-Akt, suggesting that inhibition of PKCζ activity limited its effect on Akt phosphorylation. In addition, no decrease in Akt phosphorylation was observed in EGF-treated cells transfected with dn PKCζ, compared with an empty vector control (Figure 6, middle panel). Equal transfection efficiency of dn PKCζ was determined by Western blotting of cell lysate with anti-V5 antibody (results not shown). Thus wild-type PKCζ, but not dn PKCζ, limits the EGF-induced increase in Akt phosphorylation, which is compatible with the effect of Ro-31-8220 on Akt phosphorylation (Figures 2 and 3) being due to the inhibition of endogenous PKCζ.

Wild-type PKCζ decreases LPA-induced Akt phosphorylation

LPA is a bioactive lipid exhibiting potent growth factor activity; it induces multiple biological responses in breast cancer cells [25]. In addition, LPA can induce a PI-3K-dependent activation of PKCζ and Akt through membrane-anchored G-protein-coupled receptors [26]. To determine whether the ability of PKCζ to limit the activation of Akt can be generalized to multiple growth factors and G-protein-coupled receptors as well as tyrosine-kinase-linked receptors, we co-transfected MDA-MB-468 breast cancer cells with empty vector or wild-type PKCζ with HA-Akt, and then treated cells for 10 min with 5 μM LPA. Western blot analysis with the anti-(phospho-Ser-473) antibody indicated that LPA induced a 2.2-fold increase in Akt phosphorylation over control levels (Figure 6, bottom panel). Co-transfection of wild-type PKCζ severely curtailed LPA-induced Akt phosphorylation without altering basal Akt phosphorylation (Figure 6, bottom panel). The ability of LPA to activate extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in cells expressing exogenous PKCζ and Akt was determined by Western blotting with anti-(phospho-ERK) antibody (Figure 6, bottom panel). Taken together, these results suggest that PKCζ negatively

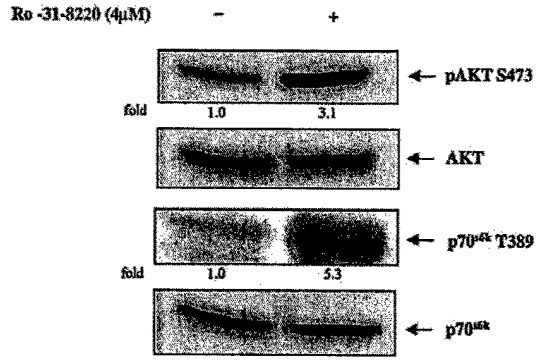

Figure 7 Ro-31-8220-induced phosphorylation of p70^{s6k} at Thr-389

BT-549 cells were starved overnight and then treated with Ro-31-8220 at 4 μM for 1 h. Cells were lysed; lysates were subjected to Western blotting with anti-(phospho-Ser-473) (Akt) or anti-(phospho-Thr-389) (p70^{s6k}) antibodies. The membrane was stripped and re-probed with anti-Akt or anti-p70^{s6k} antibodies to indicate that the levels of Akt and p70^{s6k} proteins were not changed by the treatment. Results were quantified by densitometry as described above. The results shown are representative of three independent experiments.

regulates Akt phosphorylation in a growth-factor-dependent manner.

Ro-31-8220 increases phosphorylation of Thr-389 on p70^{s6k}

Phosphorylation of Thr-389 in the linker domain between catalytic and autoinhibitory domains is obligatory for p70^{s6k} activity, whereas phosphorylation on sites located within the C-terminal autoinhibitory domain or the catalytic domain by FRAP/RAFT/mTOR, p38, PDK1 and possibly other kinases modulates the effect of phosphorylation of Thr-389 [27,28]. To determine whether the increased Akt activity associated with PKC inhibition was transmitted through the PI-3K/Akt signalling cascade, we assessed the effect of Ro-31-8220 on the phosphorylation of Thr-389 in p70^{s6k}. As indicated in Figure 7, Ro-31-8220 (4 μM, 1 h) increased Akt phosphorylation on Ser-473 in BT-549 cells concomitantly with Thr-389 in p70^{s6k}, which is consistent with increased signalling through the PI-3K/Akt signalling cascade. Again, the PKC inhibitor Ro-31-8220 did not change expression levels of Akt and p70^{s6k}.

DISCUSSION

The production of three phosphorylated phosphatidylinositols by PI-3K results in the activation and recruitment to the membrane of a subpopulation of PH-domain-containing proteins [29]. In addition, three phosphorylated phosphatidylinositols bind to and activate a number of proteins containing C2 domains, such as the PTEN tumour suppressor gene product, some phosphatidylinositol kinases (not PI-3K) and nPKCs and cPKCs, but not aPKCs, which lack the C2 domain found in other PKCs [29–31]. The aPKCs are regulated by PI-3K through binding to PDK1 [32] and a PDK1-mediated phosphorylation of a negative regulatory site in the activation loop of aPKCs [32,33]. The role of each of the PI-3K targets in the positive or negative regulation of the flow of signals through the PI-3K pathway is only beginning to be elucidated. The difficulty in analysing the integration of signals through the PI-3K cascade is magnified when cross-talk between multiple signalling pathways is taken into account.

Most studies of the PI-3K pathway have focused on the positive flow of signals through the PI-3K signalling cascade contributing to cell survival, proliferation and differentiation through Akt, Ilk, PDK1, Tec kinases and their downstream targets Bad, p70^{s6k}, caspase 9, forkhead and GSK3α/β [2,3,34,35]. However, the recent discovery that the PTEN tumour suppressor gene product, implicated in tumorigenesis in multiple cell lineages and through germline mutations in the Cowden's breast cancer predisposition syndrome, specifically dephosphorylates the same 3′ site in the inositol ring that is phosphorylated by PI-3K has focused attention on the negative regulation of this pathway [36,37]. In addition to PTEN, SHIP, by dephosphorylating the 5′ site on the inositol ring of PtdIns(3,4,5)P_3, also seems to act as a negative regulator of signalling through the PI-3K pathway [38–40].

Multiple PKC family members and in particular aPKCs, including PKCζ and PKCλ/ι, are activated as a consequence of stimulation of PI-3K activity by many different growth factor receptors [32,41]. The present studies indicate that the aPKCs, particularly PKCζ, participate in a negative feedback loop initiated by the activation of PI-3K by EGF and LPA and the subsequent activation of PKCζ, which decreases the phosphorylation and activity of Akt. This activity of PKCζ is transmitted through the PI-3K signalling cascade as a decrease in activation of p70^{s6k}. Because PKCζ and p70^{s6k} can form a physical complex [42], PKCζ might affect p70^{s6k} activity by several mechanisms. The results linking p70^{s6k} phosphorylation to Akt and PKCζ extend a previous study that suggested that PKCζ could decrease the ability of PDGF (platelet-derived growth factor) to activate Akt and transmit signals to GSK3α [10]. The consequence of the feedback loop activated by PKCζ is not clear. Although, as mentioned above, PKCζ decreases signal transduction through PI-3K and Akt to p70^{s6k} and GSK3α, which would be expected to decrease cellular proliferation and viability, PKC and probably PKCζ also alter the phosphorylation of Bad [43], which would be predicted to increase cell survival. Thus the outcome of PI-3K-induced activation of PKCζ probably depends on the intracellular machinery of the cell stimulated, the particular ligand activating the cell and the network of signalling cascades activated.

The mechanism(s) by which PKCζ regulates the phosphorylation and activation of Akt also seem complex. PKCζ interacts physically with Akt (Figure 5) [10,22,23], suggesting that Akt might be a direct target for phosphorylation by PKCζ. Intriguingly, the effects of growth factors on this association might be quite different, depending on the receptor activated or the cell stimulated, because we have demonstrated that EGF markedly increases the association of PKCζ with Akt in breast cancer cells (Figure 5), whereas PDGF has been reported to dissociate PKCζ and Akt in Cos cells [10]. Our studies with inhibitors of PI-3K indicate that the effects of PKCζ on Akt phosphorylation exhibit both PI-3K-dependent and PI-3K-independent components. The PI-3K-dependent component might indicate, at least in part, a requirement for activation of PKCζ by PI-3K through PDK1, because the overexpression of PKCζ did not alter basal Akt phosphorylation but rather blocked EGF-dependent or LPA-dependent Akt phosphorylation and activation. Doornbos et al. [10] suggest that the effects of PKCζ are directly on Akt and are independent of upstream mediators such as PI-3K or SHIP, because they are exhibited in cells transfected with a membrane-targeted and growth-factor-independent PI-3K. However, because membrane-targeted PI-3K remains subject to regulation by the p85 subunit [44], this fails to rule out the possibility that PKCζ might directly or indirectly have effects upstream of Akt. Although the physical interaction between PKCζ and Akt suggests that Akt might be a direct target of PKCζ, it is not

known whether the PI-3K-independent effect of PKCζ is solely on Akt directly or whether other signalling molecules such as adenylate cyclase [9] are involved. Thus it remains possible and even likely that PKCζ mediates multiple effects on the PI-3K signalling cascade upstream of Akt, at Akt and probably downstream of Akt.

An obligatory role of the PTEN tumour suppressor gene in the action of PKCζ and PKC inhibitors on the phosphorylation of Akt is eliminated by the observation that both of the breast cancer cell lines used in these studies lack functional PTEN owing to homozygous mutations in the PTEN gene sequence that result in a complete lack of detectable PTEN protein [37]. Similarly, an obligatory role for conventional PKC in the effects of the PKC inhibitors on Akt phosphorylation and activation seems to be eliminated by the failure of MDA-MB-468 cells to express detectable levels of conventional PKCs (Figure 1). Although a lack of conventional PKCs might be unusual, it is not entirely without precedent, because we have previously identified an interleukin-2-dependent cell line apparently lacking conventional PKC protein and enzyme activity [45].

The concentration dependence of the effects of the PKC inhibitor GF109203X, which inhibits cPKC and nPKC at nanomolar concentrations and aPKC at micromolar concentrations [18,20], suggests that aPKC might have a major role in the effects of the inhibitor Ro-31-8220, although a small but significant (approx. 3.2-fold) increase in Akt phosphorylation was observed with 0.1 μM GF109203X, indicating the possible involvement of nPKC in the regulation. Although the effects of the inhibitors implicate aPKC, as with all kinase inhibitors GF109203X and Ro-31-8220 might inhibit other kinases. However, the observations with the PKC inhibitors, when combined with the ability of PKCζ to bind Akt in a growth-factor-dependent manner and the ability of PKCζ to decrease growth-factor-induced Akt phosphorylation and activation, implicates PKCζ as a major regulator of Akt phosphorylation and activation.

In summary, we have demonstrated that Akt and PKCζ physically associate in a growth-factor-dependent manner. This association results in a decrease in the phosphorylation and activity of Akt, which is transduced through the signalling cascade as a decrease in $p70^{s6k}$ activation. Although PKCζ interacts physically with Akt, potential additional interactions and cross-talk between aPKCs and the PI-3K signalling cascade at the level of PI-3K, SHIP or downstream substrates cannot be eliminated. Taken together, the results indicate that PI-3K-dependent activation of PKCζ leads to a feedback inhibition of signalling through the PI-3K/Akt signalling cascade resulting in decreased Akt phosphorylation and activity, which in turn leads to decreased $p70^{s6k}$ and GSK3α functionality. The role of this negative feedback loop in the functional outcomes of PI-3K signalling probably represents a complex interplay of positive and negative signals initiated by the PI-3K cascade as well as other signalling cascades.

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Induced interaction of protein kinase C delta with phosphatidylinositol 3 kinase by oxidants and src kinase

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Running title: PKCδ association and regulation on PI3K

Summary

Background: An ability to couple to phosphatidylinositol 3 kinase (PI3K) is critical for multiple stimuli to regulate pleiomorphic cell functions, such as proliferation, growth and survival. The p85 regulatory subunit modulates PI3K activity through tyrosine phosphorylation dependent recruitment to signaling complexes including receptors, tyrosine kinases and adaptor proteins. Protein kinase C δ (PKCδ), belonging to the novel subclass of PKC family, is reported to be tyrosine phosphorylated *in vitro* and in intact cells. Whether tyrosine phosphorylated PKCδ might associate with the p85 regulatory subunit and modulate PI3K function has not been assessed.

Results: We demonstrated that oxidants, phorbol esters and src tyrosine kinase induced the p85 subunit of PI3K to associate with PKCδ. This interaction required src kinase-mediated tyrosine phosphorylation of PKCδ, which induced a direct physical association with the C-terminal SH2 domain of p85. The induced interaction of PKCδ with p85 was associated with altered signaling through the PI3K pathway as indicated by a decrease in src-induced AKT phosphorylation. Surprisingly, a catalytically inactive PKCδ was as effective in decreasing src-dependent AKT phosphorylation as wild type PKCδ, indicating that the mechanism is independent of PKCδ-catalyzed phosphorylation and may rather be dependent on PKCδ binding to p85.

Conclusions: The results demonstrate a direct and inducible association of PKCδ with the p85 subunit of PI3K, which is associated with a decrease in PI3K signaling, indicating a functional cross-talk between PKCδ and PI3K in signaling induced by oxidants, src and phorbol esters.

Introduction

Phosphatidylinositol-3 kinase (PI3K) contributes to multiple cell activities in normal and malignant cells, including proliferation, cell growth, survival and motility (1-3). PI3K consists of a heterodimer of a p110 catalytic subunit and a p85 or p50 regulatory subunit. The p85 subunit, which has multiple isoforms and splice variants, contains several functional protein: protein interaction domains: an amino-terminal SH3 domain, two proline rich regions, an amino-terminal SH2 domain, an iSH2 region, and a carboxyl-terminal SH2 domain (2). The p85 SH3 domain binds proline rich regions in other proteins mediating constitutive interactions. The p85 SH2 domains inducibly bind with high affinity and local sequence specificity to phosphotyrosine residues within Tyr (P)-X-X-methionine or Tyr (P)-X-X-leucine motifs, or, in some cases, other motifs (4). Multiple p85 partners have been identified, including the EGF receptor family members, the insulin receptor substrate-1, cytokine receptors, src kinase, CD28, rac1, CDC42 and the cbl adaptor protein (2,5,6). p85 regulates PI3K activation through multiple mechanisms including: (1) iSH2 mediated binding to the p110 catalytic subunit both stabilizing p110 and restricting basal p110 activity; (2) recruitment of p110 to activation complexes and juxtaposition of p110 to its membrane substrates; and (3) reversal of inhibition and potentially activation of PI3K following binding of the SH2 domains to phosphotyrosine in the appropriate context. Thus, p85 and its physical interaction with other proteins play a critical role in regulating PI3K function.

Activated PI3K phosphorylates membrane phosphatidylinositols (PtdIns) on the 3 hydroxyl recruiting a subset of proteins with pleckstrin homology (PH), Phox, FYVE, conserved 2 (C2) and conserved 1 (C1) domains to the membrane. The importance of this process is emphasized by the observation that the PTEN tumor suppressor gene dephosphorylates the same site in PtdIns phosphorylated by PI3K and that multiple components of the PI3K pathway are aberrant in cancer cells and exhibit transforming

activity (7,8,9). Biochemical and genetic studies have implicated protein kinase B (PKB)/AKT as a critical downstream mediator of PI3K. Binding of the PH domain on AKT to the lipid products of PI3K recruits AKT to the cell membrane where AKT becomes activated (10). Full activation of AKT is, at least in part, dependent on phosphorylation of Ser473 at the C-terminus and Thr308 in the catalytic domain and two tyrosines near the catalytic site (10). The actions of AKT and PI3K are realized at least in part, by its ability to phosphorylate downstream substrates including glycogen synthase kinase 3 (GSK3), p70 S6 kinase, forkhead transcription factor (FKHR), BAD, cAMP response element binding protein (CREB), BRCA1, nuclear factor-κB (NFκB), p21, p27 and potentially caspase 9 (11).

The src tyrosine kinase family is widely expressed in mammalian cells. The src family kinases are regulated by phosphorylation of Tyr416 (or its equivalent) in the activation loop of the kinase domain which activates src; whereas, the phosphorylation of Tyr527 (or its equivalent) at the C-terminal inactivates src, a process that is reversed by the SHP1 tyrosine phosphatase (5,12,13). Compatible with this, the loss of Tyr527 or mutation at this site leads to an enhanced enzymatic activity of the kinase and renders it insensitive to SHP1 (14,15). Src and other family members phosphorylate Tyr688 in the p85 subunit releasing the inhibitory activity of p85 on p110 (5). Src can also activate PI3K through the phosphorylation of other mediators, such as receptor tyrosine kinases and linker molecules, which, in turn, interact with the p85 subunit of PI3K resulting in activation of p110. v-src transformed cells display a variety of aberrant properties including transcription of genes involving mitogen signaling, abnormal cell cycle and differentiation, and prevented cell death and migration. Further *in vivo* src activity has been linked to the growth of many types of tumors including breast cancer (16). Consistent with this, src inhibitors can suppress tumor growth in animal models (17).

The protein kinase C (PKC) family of serine/threonine kinases can be subdivided into three classes: classic PKCs, novel PKCs and atypical PKCs according to their regulation by different mediators (18). Phosphorylation at specific serine or threonine residues is critical for the functional activities of most PKC enzymes (19). For PKCδ, a member of the novel PKC subfamily, phosphorylation of Thr505 in the activation loop of the kinase domain and of Ser643 at the C-terminal correlate with its enzyme activity. Furthermore, PKCδ, θ, α and ε can be tyrosine phosphorylated (20-22). Phorbol esters, growth factors, oxidants and oncogenes are all able to induce tyrosine phosphorylation of PKCδ albeit, potentially on different residues. The impact of PKCδ tyrosine phosphorylation on cell physiology remains unclear. PKCδ activation is involved in the apoptosis induced by tumor necrosis factor α (TNFα), anti-Fas-antibody and radiation (23). Consistent with the capacity of PKCδ to induce cell death, transgenic mice overexpressing PKCδ demonstrate a dramatic reduction in tumor growth upon treatment with phorbol 12-myristate-13-acetate (PMA) (24). Thus, PKCδ possesses ability to suppress tumor progression dependent on both its activity and the interaction with other cellular signal proteins.

Both environmental exposure and inflammation generate a variety of oxidants. Oxidative stress, in turn, stimulates signal flows to regulate cell activities, including growth and proliferation (25,26). Oxidants are able to induce phosphorylation and activity of membrane receptors subsequently leading to activation of multiple signal proteins including PKC and mitogen-activated protein kinase (MAPK) (26-28). In the view of the impact on cell physiology, oxidants are implicated in both initiation and development of many different types of tumors, such as lung cancer and head and neck cancer (29,30). This largely depends on the cellular signaling processes due to changes in redox status. Moreover, overexpression of H_2O_2-generating enzymes leads to formation of tumor in nude mice (31), further supporting the role of oxygen metabolites in tumorigenesis. The

mechanisms by which oxidative stress contributes to malignant growth are currently unclear.

We demonstrated, herein, that oxidants, src kinase and phorbol esters induce a direct physical interaction between PKCδ and the p85 subunit of PI3K. The association requires tyrosine phosphorylation of PKCδ and selectively involves the carboxyl-terminal SH2 domain of p85. The interaction of PKCδ with p85 leads to a reduction in src-induced and PI3K-mediated AKT phosphorylation. Together, these findings implicate a cross-talk between PKCδ and PI3K in signaling initiated by a variety of stimuli.

Results

Oxidative stress induces association of PKCδ with p85. Multiple PKC isoforms are functionally responsive to redox modification (31). H_2O_2 stimulates tyrosine phosphorylation of PKCδ (33,34), which is mediated by the activity of src family tyrosine kinases. Also, the observations that PKCδ can be tyrosine phosphorylated, and that PI3K associates with phosphorylated proteins including receptors, kinases and adaptors suggest the possibility that PKCδ may associate with PI3K either directly or indirectly altering PI3K function. To clarify this issue, we examined whether H_2O_2 could induce formation of a complex containing PKCδ and p85. We transiently stimulated Cos7 cells with 2mM H_2O_2, lysed cells and then immunoprecipitated cellular PKCδ with an anti-PKCδ antibody. Precipitated proteins were separated on SDS-PAGE and the association of p85 with PKCδ was monitored by western blot with anti-p85. As shown in Fig. 1A, p85 was readily detected in PKCδ immunoprecipitates from H_2O_2 -treated cells but not resting cells. Concordant with H_2O_2-induced increase in the association of p85 with PKCδ, H_2O_2 increased PKCδ tyrosine phosphorylation (Fig.1A). A non-immune serum failed to precipitate PKCδ (data not shown). In addition, following stimulation and lysis we precipitated the endogenous p85 from cell lysates with anti-p85 antibody, and detected the presence of PKCδ in the precipitates by anti-PKCδ blot. The result, consistent with the previous observation, demonstrated that PKCδ and p85 associate in response to oxidant stimulation (Fig. 1A). A non-immune serum was included in the experiment as a negative control. Thus, H_2O_2 is sufficient to induce an interaction of PKCδ with p85, a process potentially dependent on tyrosine phosphorylation of PKCδ.

PKCδ does not possess SH2 or PTB phosphotyrosine binding domains suggesting that the SH2 domains of p85 may mediate the interaction of p85 with PKCδ (35). Further PKCδ contains two YxxL consensus motifs located within its catalytic domain that if phosphorylated could mediate binding by p85 SH2 regions (20). To further investigate this

possibility, we transiently transfected Cos7 cells with V5-tagged PKCδ and either the carboxyl-terminal SH2 domain (CSH2) or the amino-terminal SH2 domain (NSH2) of p85 epitope tagged with HA. After transfection, cells were starved overnight prior to stimulation with H_2O_2. The HA-tagged SH2 peptides were then immunoprecipitated and the presence of V5PKCδ in the complex was detected by blotting with anti-V5. As indicated in Figs. 1B and 1C, H_2O_2 induced a marked increase in binding of V5PKCδ to the C-terminal SH2 domain of p85 but not the N-terminal domain. Moreover, pretreatment with the PP1 src inhibitor reduced H_2O_2-stimulated association by over 50% as indicated by densitometry. Western blotting with anti-pTyr antibody revealed that in response to H_2O_2 the PKCδ bound to p85 was tyrosine phosphorylated (Fig.1B), consistent with the involvement of tyrosine phosphorylation in the capacity of PKCδ to bind p85. Furthermore, the phosphorylation was decreased by PP1 compatible with the reduced association of PKCδ with p85 (Fig.1B). Taken together, oxidants stimulate the formation of a complex between PKCδ and the CSH2 domain of p85, a process that is regulated by src family kinase activity and PKCδ tyrosine phosphorylation.

PMA induces a src-dependent association of PKCδ with p85. PMA not only activates PKCδ but also induces PKCδ tyrosine phosphorylation (Fig. 2A) (36). To evaluate the role of src activity in PMA-mediated PKCδ tyrosine phosphorylation, we assessed the effect of pretreatment of MDA-MB-468 cells with the src inhibitor, PP1. The increase in PKCδ phosphotyrosine level induced by PMA was reduced to baseline levels by preincubation with PP1 (Fig. 2A), indicating that src is required for PMA-induced PKCδ tyrosine phosphorylation. Based on our findings presented so far showing that the p85 CSH2 domain binds tyrosine phosphorylated PKCδ, we assessed whether PMA could also promote the interaction of PKCδ with p85. Notably, PMA treatment induced association of PKCδ with the CSH2 domain of p85 but not the NSH2 domain (Figs. 2B, 2C). PMA also induced association of PKCδ with a NiC construct (Fig. 2B) that contains the CSH2

domain along with the NSH2 and the iSH2 domains. The ability of the CSH2 domain to bind PKCδ as efficiently as the NiC construct strongly argues that the NSH2 region does not alter the interaction. Taken together, the data indicates that PMA treatment is associated with a series of subsequent events including src-dependent PKCδ tyrosine phosphorylation and association with the p85 subunit of PI3K.

The p85 carboxyl-terminal SH2 domain binds to PKCδ in the presence of activated src. Src has been implicated as a target of multiple stimuli as well as in multiple cancer lineages and we have implicated src family kinases in the regulation of the PI3K signaling cascade at multiple levels (37). As indicated above, agonist-induced p85 association with PKCδ was modified by inhibition of the activity of src family kinases. We, thus, assessed the effect of a constitutively activated form of src (Y527F) tyrosine kinase, which was generated through mutating the C-terminal inhibitory tyrosine to phenylalanine, on the association of PKCδ with p85. For this purpose, V5PKCδ and HAp85 were transiently overexpressed in Cos7 cells. As demonstrated in Fig. 3A, src Y527F induced a dramatic increase in the association of HAp85 with V5PKCδ in comparison with a relatively weak basal interaction seen in control cells. Note that coexpression of activated src with PKCδ induced a size shift compatible with phosphorylation of PKCδ (Fig. 3A) (38). Thus activated src recapitulates the effects of oxidative stress and effectively induces interaction of PKCδ with p85.

To evaluate whether the src-dependent association of PKCδ with p85 is mediated by a phosphotyrosine-SH2 interaction, we examined the effect of src on the ability of V5PKCδ to bind to the carboxyl- or amino- SH2 domains of p85. As shown in Fig. 3B, results of this analysis revealed a src-dependent association of the CSH2 with PKCδ. In contrast, although highly expressed in transfected cells, the NSH2 domain failed to bind to PKCδ (Fig. 3B). The expression level of PKCδ in each transfection was determined by western

blotting the whole cell lysates with anti-V5 mAb. A non-specific immune blot was indicated which migrated at a distinct rate from PKCδ.

Src induces tyrosine phosphorylation of PKCδ. As indicated in Fig.3, src induces a size shift in PKCδ compatible with increased tyrosine phosphorylation. Further the src-induced interaction of PKCδ with the C-terminal SH2 domain suggests that tyrosine phosphorylation of PKCδ is most likely involved in the binding of PKCδ with p85. We examined the effect of src on PKCδ tyrosine phosphorylation. As indicated in Fig.3C, src induced tyrosine phosphorylation of PKCδ primarily but not solely on the slower migrating form. As we have shown previously, coexpression of src and the CSH2 domain of p85 resulted in tyrosine phosphorylation of the CSH2 domain, a process we have shown to involve Tyr688 (5).

p85 directly binds tyrosine phosphorylated PKCδ. As described above, activated src induces association of tyrosine phosphorylated PKCδ with p85, specifically the CSH2 domain. This could be due to the direct interaction of the CSH2 domain of p85 with a phosphotyrosine in PKCδ. Alternatively, intermediary molecules may contact both p85 and PKCδ. To assess the hypothesis that the interaction was direct, we utilized " Far Western " or " Protein Overlay" blotting. As indicated in Fig.4A, the carboxyl-terminal SH2 domain of p85 directly bound to both epitope-tagged and non-tagged PKCδ. The interaction was markedly increased when PKCδ was immunoprecipitated from cells expressing activated src. Thus optimal binding of the CSH2 domain of p85 to PKCδ requires tyrosine phosphorylation of PKCδ but not tyrosine phosphorylation of the p85 SH2 domain. Again, a non-specific immune blot was observed with a distinct migrating rate compared to V5PKCδ.

We also performed the Far Western assay in the reverse direction by precipitating HAp85 or SH2 domains of p85 from cells with and without activated src, followed by probing with recombinant non-phosphorylated PKCδ. The recombinant PKCδ demonstrated a weak interaction with full-length p85 and the CSH2 domain of p85 but not the NSH2 domain (Fig. 4B). Transfection of activated src, which induces tyrosine phosphorylation of p85 in its CSH2 domain, failed to alter the interaction (Fig. 4B), in contrast to the induced binding of the CSH2 domain of p85 to tyrosine phosphorylated PKCδ (Fig. 4A). Thus, tyrosine phosphorylation of PKCδ plays a major role in mediating a direct physical association with the CSH2 domain of p85.

PKCδ decreases src-induced AKT phosphorylation. To determine whether the association of PKCδ with the p85 subunit of PI3K affects PI3K function, we analyzed the effect of PKCδ on src-stimulated AKT phosphorylation as an indicator of functional signaling through PI3K activity. As demonstrated in Fig.5A, src Y527F induced a marked increase in AKT phosphorylation (note the total AKT antibody used in this study does not react efficiently with phospho-AKT accounting for the apparent decrease in total AKT levels). By contrast, when PKCδ was also coexpressed with src and AKT, a decreased AKT phosphorylation was detected, reflecting a negative effect of PKCδ on signaling by src through PI3K to AKT. The use of a constitutively activated src construct supports the contention that the action of PKCδ is at the level of PI3K rather than on src. PKCδ alone did not modulate AKT phosphorylation, further suggesting that the effect of PKCδ on AKT phosphorylation is related to src-dependent activation of PI3K. To assess whether the enzymatic activity of PKCδ is essential in altering signal transduction through PI3K, we employed a catalytically inactive mutant of PKCδ (dn PKCδ), which contains a K378R point mutation at its ATP binding site leading to a loss of its catalytic function (39). As seen in Fig. 5A, the catalytically inactive PKCδ reduced src-mediated AKT phosphorylation to a similar extent, as did wild type PKCδ. Comparable transfection efficiency of both src

Y527F (Fig. 5A) and PKCδ, either wild type or mutant (data not shown), were observed. This suggests that PKCδ modulates PI3K signaling through a non-phosphorylation dependent mechanism. Supportive of a lack of a role for the enzymatic activity of PKCδ in altering AKT phosphorylation, coexpression of activated src failed to enhance PKCδ phosphorylation at Ser643 and Thr505 (Fig. 5B), suggesting that src does not activate PKCδ. Phosphorylation of these residues is required for optimal PKCδ activity (19).

Discussion

In this paper, we have demonstrated a direct physical interaction between tyrosine phosphorylated PKCδ and the CSH2 domain of the regulatory subunit, p85, of PI3K. This process is induced by different activators including oxidative stress, src and PMA. Further this response can be demonstrated between endogenous as well as overexpressed proteins. Src is sufficient to induce the association of PKCδ with p85 and is required for optimal PMA- and H_2O_2-induced association. The induced interaction is dependent on direct binding of the carboxyl terminal SH2 domain of p85 with tyrosine phosphorylated PKCδ possibly at one of the YxxL motifs. However, we can not exclude the potential involvement of other tyrosine-containing contexts of PKCδ, for example, tyr187 and tyr311 that are phosphorylated following treatment of cells with PMA and oxidants respectively (34,40). p85 SH2 domains are able to recognize divergent sequences at least in model systems (4). Thus, it will be interesting to determine which tyrosines are able to bind p85 and whether the interaction regulates PKCδ enzyme activity and biological functions.

In contrast to our data, a previous report of Ettinger et al has shown a PKCδ and PI3K association that is independent of tyrosine phosphorylation (41). This may represent the low basal interaction between PKCδ and p85 as well as the low-level interaction of PKCδ with the CSH2 domain of p85 we observed in PKCδ overlay assays (Fig.3). It is important to emphasize the different experiment conditions: different cell systems and stimuli. In TF-1 cells, Ettinger et al have observed that cytokines induce binding between PKCδ and p85 but not tyrosine phosphorylation of PKCδ indicative of a tyrosine phosphorylation-independent association. This is obviously different from the src, PMA, and H_2O_2 induced phosphotyrosine-SH2 mediated association described herein. Therefore, it appears that PKCδ and p85 interaction may occur through different mechanisms dependent on the signaling systems engaged in the cells.

Our data have indicated that PKCδ preferentially binds to the CSH2 domain of p85 as compared to the NSH2 domain. This is consistent with the previous literature of differential binding ability of the two domains (42). The molecular basis for the differential binding has been revealed through mutation of cys57 in the CSH2 domain to isoleucine (Ile), the corresponding residue in the NSH2 domain, resulting in a shift of selectivity of the CSH2 domain towards to that of NSH2 (5,43). The possibility that the two SH2 domains cooperate in binding PKCδ appears unlikely because PKCδ binds equally well to CSH2 as to a peptide including both CSH2 and NSH2 following PMA treatment.

PKCδ, which has been proposed to demonstrate tumor suppressor properties (44,45), plays an important role in the inhibition of cell growth and promotion of cell death. PKCδ is proteolytically cleaved by caspase 3 generating an active catalytic 40 kDa kinase domain fragment, which catalyzes phosphorylation in serine/threonine residues of substrates contributing to caspase-mediated cell death (46-48). Several nuclear targets of PKCδ have been found, including proteins involved in DNA repair, DNA-PK, and maintenance of nuclear structure, lamin B (3,49,50). We, in contrast, in the current study demonstrate that PKCδ is capable of reducing src-induced AKT phosphorylation. As AKT is a major contributor to the ability of PI3K to prevent apoptosis, this may represent a novel role of PKCδ in suppressing cell survival. Furthermore, the similar behavior of the catalytic inactive PKCδ as the wild type PKCδ on AKT phosphorylation argues against a role of enzymatic activity of PKCδ in the process. This further distinguishes the event from PKCδ activity and substrate phosphorylation implicated in the contribution to caspase-induced apoptosis.

The mechanisms by which PKCδ interferes with src-induced PI3K activity and signaling remain to be determined. Src induces PI3K activity through generating SH2-phosphotyrosine interactions between the p85 subunit and signal proteins (13), which, in

turn, facilitates an active conformation for PI3K. We have also shown that src kinases can relieve the inhibitory effect of p85 on p110 by the formation of an interaction between phospho-Y688 and the NSH2 domain (5). Based on this data, one model is thus established to describe the PKCδ-mediated inhibition of PI3K: PKCδ competes for binding to the SH2 domain of p85 with other low affinity p85 partners thus preventing PI3K from activation. This appears to be probable because many PI3K activation proteins, such as Gab1 and Grb2, target p85 SH2 domains (12). Alternatively the interaction of PKCδ with p85 could alter PI3K recruitment to the cell membrane or access to substrates.

Both oxidants and phorbol esters serve to promote tumor development by increasing activity of signaling pathways involved in growth control (32,51). PKCs are major cellular targets for activation by tumor-promoting agents and thus important in tumor growth. However, recent studies have revealed differential functions of PKC isoforms in cell growth and survival, suggesting that tumor promotion is linked to a subpopulation of PKC isoforms. PKCδ, in most cases, acts as a tumor suppressor. PKCδ is activated directly and phosphorylated indirectly through kinases following addition of phorbol esters and oxidants (32,38). However, the role that activation and phosphorylation of PKCδ plays in downstream signaling by these stimuli is unclear. Herein, we have demonstrated that H_2O_2, PMA and src induce PKCδ to associate with PI3K. This action is further manifest as a decrease in AKT phosphorylation compatible with functional inhibition of signal transmission through the PI3K pathway. Whether the interaction of PKCδ with the PI3K signal pathway alters the influence of oxidants on activities of other cellular molecules, signal transfer within and between signaling cascades, and overall cell activities, such as growth and survival, require further investigation. Moreover, it is possible that additional PKC isoforms which are tyrosine-phosphorylated by the action of phorbol esters, oxidants or both may interact with PI3K, affecting its function and regulating signal transduction.

Conclusions

In this paper, we have reported that PKCδ directly associates with the p85 regulatory subunit of PI3K. The interaction is inducible by multiple agonists including oxidants, src kinase and phorbol esters. PKCδ association with p85 requires src-dependent tyrosine phosphorylation of PKCδ and specifically targets the C-terminal SH2 domain of p85. Furthermore, physical interaction of the proteins is accompanied by a limited signaling through PI3K as indicated by decreased AKT phosphorylation. Independence of the process on the enzymatic activity of PKCδ suggests a mechanism not requiring ser/thr phosphorylation induced by PKCδ but rather involving association of PKCδ with p85.

Experimental Procedures

Antibodies and Reagents. The murine anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA). The monoclonal anti-HA antibody was a kind gift from Dr. Bing Su (University of Texas at Houston). The rabbit anti-PKCδ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-phospho-AKT, anti-AKT, anti-pS643 and anti-pT505 antibodies were from Cell Signaling Technology Inc. (Beverly, MA). The anti-pTyr antibody, recombinant PKCδ and recombinant carboxyl-terminal SH2 domain of p85 were purchased from Upstate Biotechnology (Lake Placid, NY). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from BioRad (Hercules, CA). PMA and H_2O_2 were from Sigma (St. Louis, MO). The cDNA plasmid of V5PKCδ was from Invitrogen (Carlsbad, CA). cDNA plasmids of PKCδ and dn PKCδ were generous gifts from Dr. Xinmin Cao (National University of Singapore, Singapore). The HAp85 and derived partial p85 fragments were generous gifts from Dr. Thomas Mustelin (La Jolla Cancer Research Center, The Burnham Institute, La Jolla, CA). HA-epitope tagged AKT was a generous gift of Dr. Julian Downward (London, UK).

Cell lines. Cos7 from American Type Culture Collection (Rockville, MD) and human breast cancer cell line MDA-MB-468 (UT MD Anderson Cancer Center, Houston, TX) were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing penicillin/streptomycin (1%, GIBCO), L-glutamine (2mM, GIBCO), and 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere.

Transient transfection. Cells were grown in 100 mm cell culture dish until 80% confluence before transient transfection with various combinations of the expression

vectors as indicated using the Fugene^{TM} 6 Transfection Reagent (Boehringer Mannheim Inc., Indianapolis, IN) as recommended by the manufacturer.

Immunoprecipitation and western blot. 24 hr after transfection cells were serum-starved overnight prior to treatment with H_2O_2 (2mM) or PMA (100nM) for 10 minutes. Cells were washed twice with cold PBS and lysed in ice-cold lysis buffer (50mM Hepes, pH7.4, 150mM NaCl, 1mM EGTA, 100mM NaF, 1.5mM $MgCl_2$, 10mM Na pyrophosphate, 1% Triton X-100, 1mM Na_3VO_4, 10% glycerol, 1mM PMSF and 10μg/ml aprotonin). Cellular protein concentration was determined by BCA reaction (Pierce, Rockford, IL). For immunoprecipitation, detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-V5, anti-PKCδ) for 2 hours. Immune complexes were captured by protein A/G sepharose beads as appropriate. Immunoprecipitates were washed with IP wash buffer (0.5% Triton X-100, 0.5% NP-40, 10mM Tris/HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM Na_3VO_4, 1mM PMSF). Proteins were separated by SDS-PAGE and transferred to immobilon membranes (Millipore, Bedford, MA), and immunoblotted with antibodies following the protocols provided by the manufactures. Proteins were visualized by enhanced chemiluminescence detection (ECL, Amersham).

Far Western analysis. V5PKCδ, PKCδ, HAp85 or p85 SH2 domains were immunoprecipitated from cell lysates, separated by SDS-PAGE and transferred to immobilon membranes. The blots were blocked in 5% non-fat dry milk for 1 hr at room temperature and then incubated with 0.5-1μg of the appropriate recombinant proteins overnight at 4°C. Washed blots were subject to standard western blotting assay and developed by ECL.

Acknowledgements

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Figure Legends

Fig.1. Oxidative stress induces association of PKCδ with p85. *A*, Endogenous PKCδ was precipitated from lysates after 2mM H_2O_2 treatment, and the association of p85 was examined by western blot. pTyr level of PKCδ was measured with anti-pTyr mAb. Equal loading of samples was determined by western blot with anti-PKCδ. In addition, endogenous p85 was precipitated from lysates after stimulation and the association of PKCδ was examined by western blot with anti-PKCδ antibody. *B.* After transfection and starvation, Cos7 cells were preincubated with 5μM PP1 for 40 min followed by stimulation with 2mM H_2O_2 for 10 min. HACSH2 was immunoprecipitated and the presence of V5PKCδ in the precipitates was examined with anti-V5 antibody. The numerical labels represent densitometry of the blot with control lane arbitrarily set as 1.0. Tyrosine phosphorylation of bound PKCδ was detected with anti-pTyr antibody. Equal expression of exogenous proteins was determined by western blotting the total cell lysate with appropriate antibodies. Arrows indicate location of PKCδ. *C.* HANSH2 and V5PKCδ were cotransfected into cells followed by stimulation with H_2O_2. After lysis and immunoprecipitation, V5 blot was performed to examine the level of V5PKCδ. The arrow indicates location of PKCδ. Data were representative of three independent experiments.

Fig. 2. PMA induces a src-dependent association of PKCδ with p85. *A*, After starvation for overnight, MDA-MB-468 cells were preincubated with 5μM PP1 inhibitor for 40 min, followed by stimulation with 100nM PMA for 10 min. PKCδ was precipitated, separated on SDS-PAGE and transferred onto immobilon membranes. pTyr level of PKCδ was examined as described above. *B* and *C*, after transfection with various HA-tagged p85 fragments as indicated, Cos7 cells were starved and treated with 100nM PMA for 10 min and subject to lysis and western blot to examine the association of V5PKCδ. Equal expression levels of exogenous proteins were determined by western blotting with appropriate antibodies. Arrows indicate the position of PKCδ.

Fig. 3. The p85 carboxyl-terminal SH2 domain binds to PKCδ in the presence of activated src kinase. *A*, Cos7 cells were cotransfected with HAp85 and V5PKCδ with or without src Y527F. Lysates were immunoprecipitated with anti-HA antibody, followed by V5 immunoblot. Transfection efficiency of HAp85 and V5PKCδ was determined by western blot with appropriate antibodies. The open arrowhead indicates the tyrosine-phosphorylated PKCδ, and the closed arrowhead indicates the non-phosphorylated form. *B*, The HA-tagged carboxyl- or amino-terminal SH2 domain of p85 (HACSH2 and HANSH2 respectively) was expressed in Cos7 cells as indicated. Coprecipitation of p85 SH2 domains with V5PKCδ was analyzed as above. Arrows indicate location of PKCδ. *C*, V5PKCδ was expressed in cells with either empty vector or src Y527F, precipitated from cell lysates with anti-V5 antibody followed by western blot with anti-pTyr antibody. Arrows indicate location of PKCδ. Data are representative of four independent experiments.

Fig. 4. p85 directly binds to tyrosine phosphorylated PKCδ. *A*, V5PKCδ and PKCδ plasmids were separately transfected into Cos7 cells with or without src Y527F. After immunoprecipitation of PKCδ from cell harvest, Far Western analysis using purchased proteins was performed as described in Materials and Methods (upper panel). pTyr level of PKCδ in various samples was examined by western blotting with anti-pTyr antibody (middle panel). Expression levels of exogenous PKCδ were determined by western blot with anti-PKCδ antibody (lower panel). The open arrowheads indicate location of PKCδ. The molecular weight (kD) is marked on the right. *B*. HAp85, HACSH2 and HANSH2 were overexpressed in cells as indicated and subject to Far Western analysis using recombinant PKCδ. The open arrowheads indicate position of PKCδ. Molecular weights are marked on the right side of blots.

Fig. 5. PKCδ decreases src-induced AKT phosphorylation. *A*, HAAKT in combination with various constructs was transfected into Cos7 cells as indicated. HAAKT was immunoprecipitated with anti-HA antibody, western blotted with rabbit anti-phospho-AKT (upper panel). The AKT blot examined the expression levels of exogenous AKT in the test samples (middle panel). The numerical labels represent densitometry of the blot after normalized to the total AKT level. HAAKT transfection alone was arbitrarily set as 1.0. Equal expression levels of src Y527F were determined by western blotting the whole cell lysate with anti-src antibody (lower panel). *B*, V5PKCδ was overexpressed in cells with or without src Y527F, precipitated from lysates and western blotted with anti-pS643 or anti-pT505 antibodies. Equal loading of samples was determined by V5 reblot. The open arrowheads indicate location of tyrosine-phosphorylated PKCδ, and the closed arrowheads indicate the location of non- tyrosine phosphorylated PKCδ. Data were representative of three independent experiments.

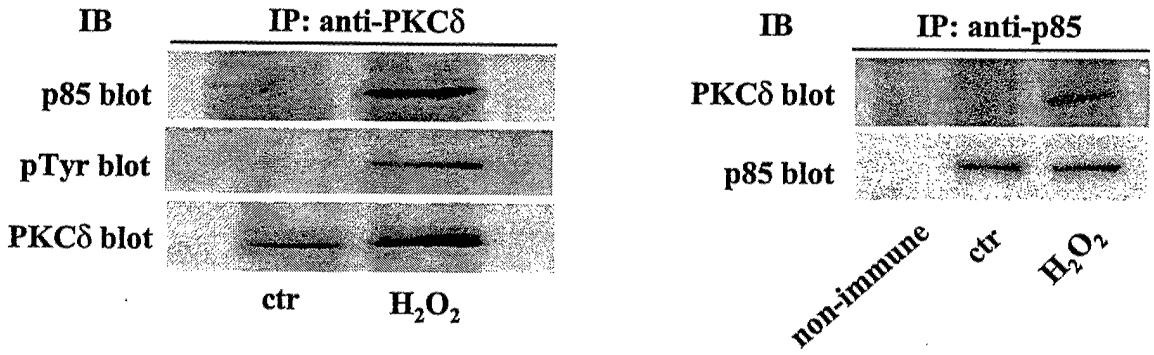
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Fig. 1A

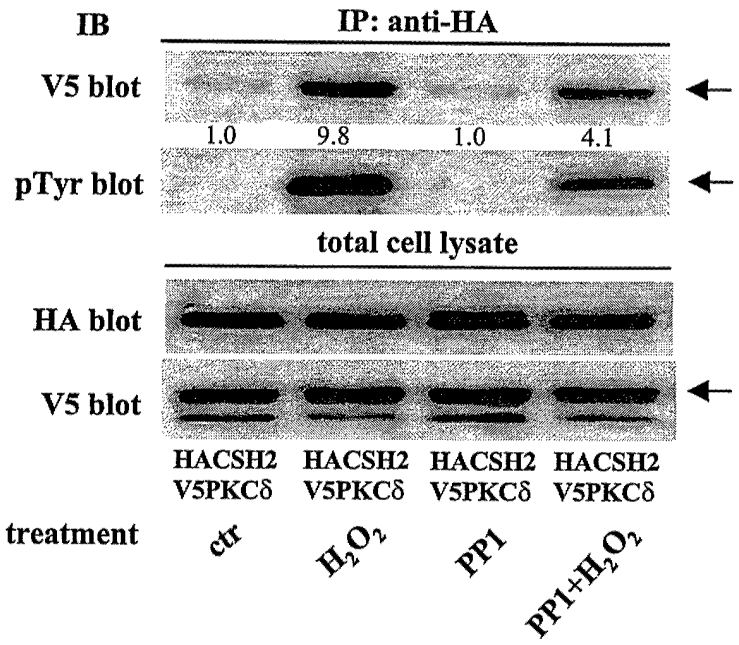
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Fig.1B

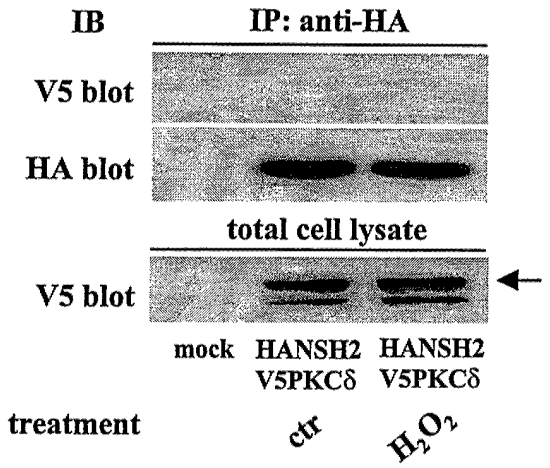
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Fig.1C

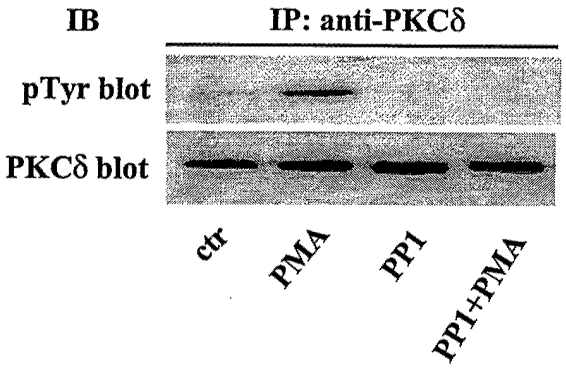
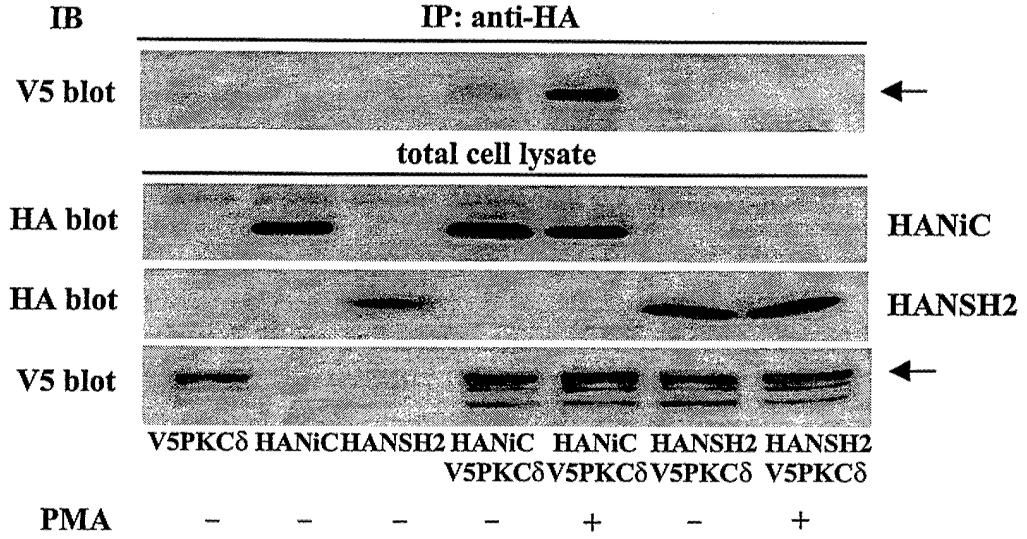


Fig. 2A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PMA
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Fig. 2B

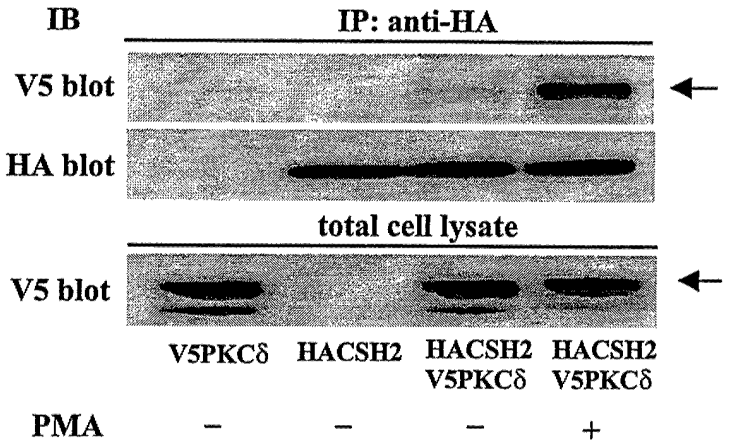
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Fig. 2C

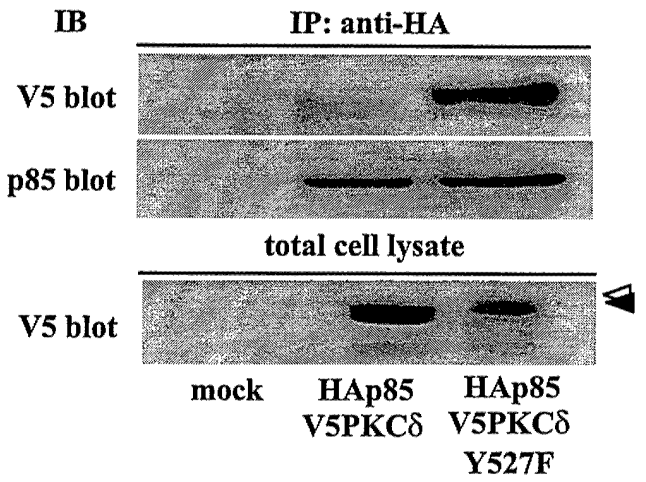
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Fig.3A

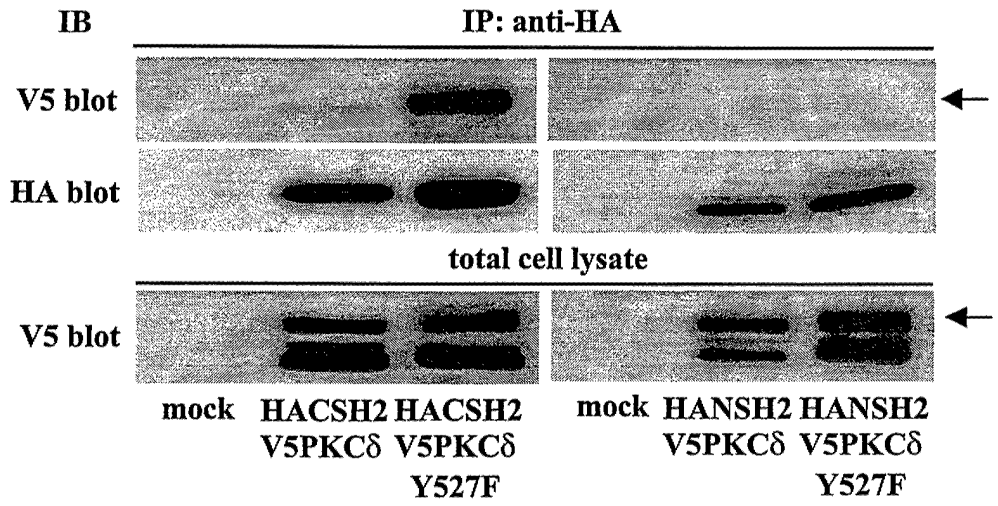
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Fig. 3B

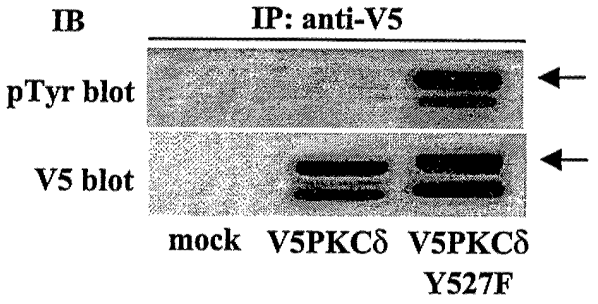

Fig.3C

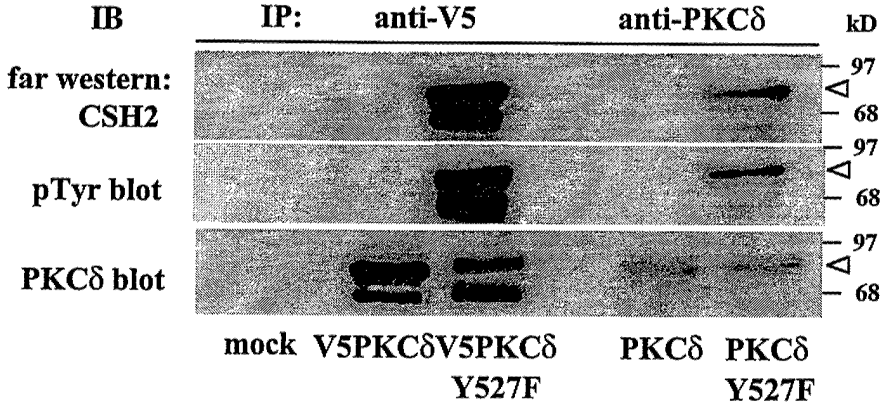
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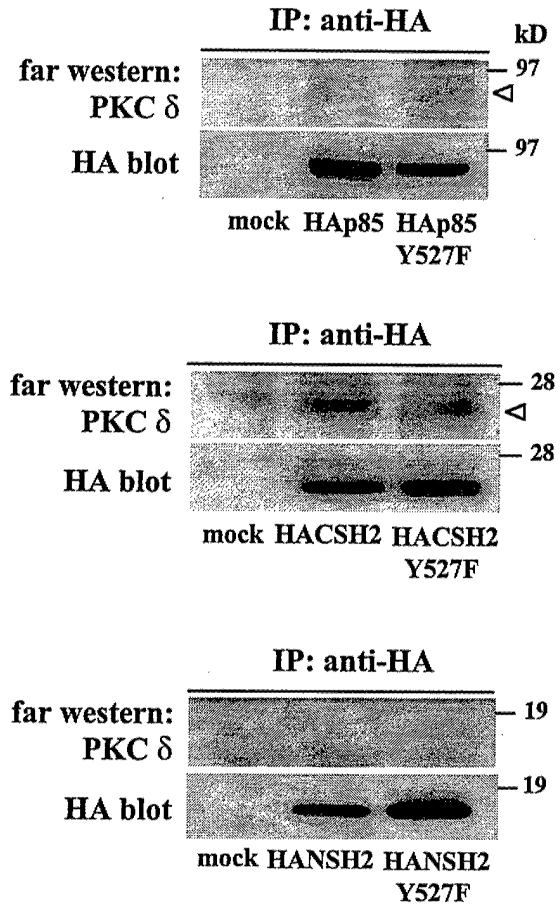
Fig. 4A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mock HACSH2 HACSH2
Y527F
IP: anti-HA
far western:
PKC δ
19
HA blot
19
mock HANSH2 HANSH2
Y527F

fig 4B

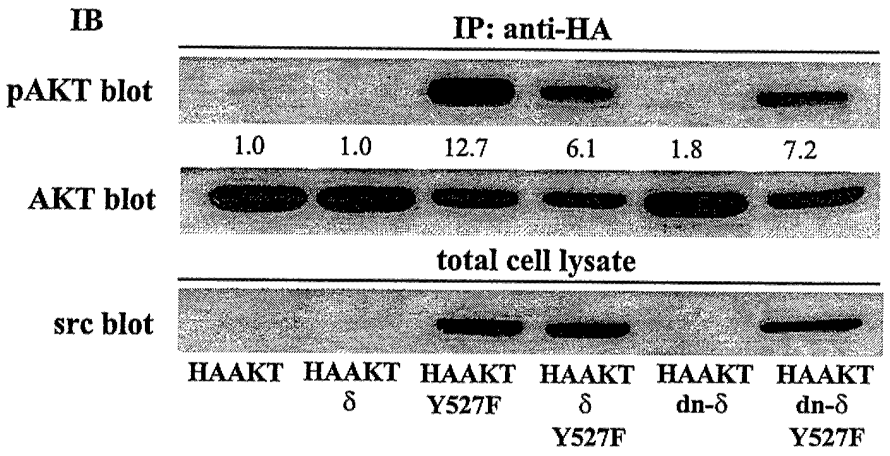
IB
IP: anti-HA
pAKT blot
1.0
1.0
12.7
6.1
1.8
7.2
AKT blot
total cell lysate
src blot
HAAKT
HAAKT δ
HAAKT Y527F
HAAKT δ Y527F
HAAKT dn-δ
HAAKT dn-δ Y527F

Fig. 5A

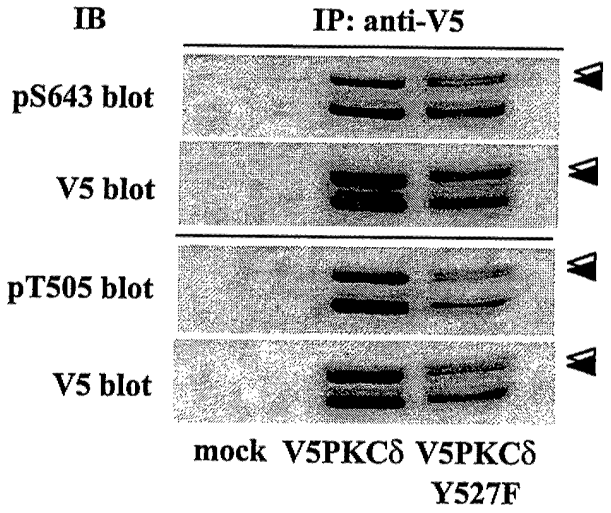
IB
IP: anti-V5
pS643 blot
V5 blot
pT505 blot
V5 blot
mock
V5PKCδ
V5PKCδ Y527F

Fig. 5B